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Novel approaches for ungual and trans-ungual delivery of drugs

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NOVEL APPROACHES FOR UNGUAL AND TRANS-UNGUAL DELIVERY OF DRUGS

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ABSTRACT

Topical therapy is commonly used for the treatment of nail diseases such as onychomycosis and nail psoriasis due to less severe side effects, non-invasiveness, direct drug delivery at the site of infection, patient compliance and lower cost of treatment. However, topical treatment is limited due to poor permeability and hard nature of nail plate.

The main objective of first project was to investigate the penetrability of apremilast into and across the nail plate, followed by the preparation of a novel nail lacquer formulation to improve its ungual and trans-ungual permeation for the treatment of nail psoriasis. Dexpanthenol and salicylic acid were found to be the potential permeation enhancers. The final nail lacquer formulation showed the ability to form a water resistant film on nail plate and delivered significant amount of apremilast into the nail apparatus.

In second project, iontophoresis technique was investigated to improve the ungual and trans-ungual permeation of itraconazole for the treatment of onychomycosis. *In vitro* and *ex vivo* permeation studies were performed following two protocols, a) pulse application of iontophoresis for 3 days (8 h/day), b) continuous application of iontophoresis for 24 h. The results of *in vitro* and *ex vivo* studies demonstrated the feasibility of iontophoresis technique to enhance nail delivery of itraconazole. Pulsed application of iontophoresis was found to be superior over continuous application of iontophoresis in all cases.

In third project, a novel hyponychium pathway was investigated to deliver drugs directly into the ventral layer of nail plate and other part of nail apparatus using iontophoresis as an active

technique. *In vitro* and *ex vivo* studies resulted that hyponychium pathway can be a viable option to deliver significant amount of drug into nail apparatus by the application of iontophoresis technique.

In final project, pre-treatment with solid microneedle was investigated to improve the availability of drugs in nail apparatus. The application of microneedles has not been explored before to improve nail delivery of drugs. Microneedle pre-treatment is a novel approach which has already been used to deliver small and large molecules into the skin in a minimally invasive manner.

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DEDICATION

This work is dedicated to my family, teachers and friends

ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my advisor, Dr. S. Narasimha Murthy for his support, patience and encouragement throughout my research work. His motivation and support helped me a lot to learn many aspects required to achieve high standards in research.

I would like to have this opportunity to express my sincere thanks to Dr. Michael A Repka, Dr. Seongbong Jo, and Dr. Dhammika Nanayakkara for accepting my request and providing their valuable time and suggestions during my prospectus and dissertation. I am thankful of my lab members (Purnendu kumar sharma, Vijay Shankar, Srinatha Aneguntha and Murali Angamuthu), specially Abhishek Juluri and Prashanth Manda who helped and supported me a lot during my graduation. I also want to thank all of graduate students of pharmaceutics and drug delivery department for their support in various projects.

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Most importantly, I would like to thank my father (Dr N.P Patel) and mother (Maya Patel) for their support and encouragement at every moment of life. A special thanks to my wife (Sonal Mourya), brother (Abhishek Kushwaha) and sister (Madhvi Kushwaha) for their loving support.

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CHAPTER 1: INTRODUCTION

Onychomycosis is a fungal infection in the toe and finger nails that is mainly caused by *Trichophyton rubrum* and *candida* (dermatophytes).[1] The fungus mainly invades and colonizes in nail plate, nail bed and matrix. It commonly starts from the hyponychium and distal or lateral nail bed and then spread all over the nail apparatus.[2] Approximately, 2-13% of general population is affected by onychomycosis.[3] People who suffer from conditions such as diabetes, peripheral vascular diseases and immunodeficiency disorders have been reported to have more vulnerability to onychomycosis. It also affects patient's emotional, social and occupational life.[3]

Nail psoriasis is a chronic inflammatory disease. It occurs approximately 50% of the skin psoriatic patients.[4] The common symptoms of psoriatic nail are pitting, onycholysis, discoloration, subungual hyperkeratosis, splinter hemorrhages. Although the theory behind the nail psoriasis occurrence is still not clear, its development is speculated due to the combination of genetic, immunologic and environmental factors.[5-7]

The treatment of nail diseases via systemic administration of drugs is not completely successful due to poor blood circulation into the nail apparatus.[3,8-10] Topical treatment is a emerging therapy which is considered to be most effective way of treatment of nail disease due to non-invasiveness, targeted drug delivery, fewer side effects, cost effectiveness and patient compliance.[3] However, poor permeability of nail plate limits the topical delivery of drugs.[2,3] Nail plate is known to possess three layers which are dorsal, intermediate and ventral.[1,2] Ventral layer is directly attached to the nail bed and relatively more hydrated than other two layers. According

to the previous literature, dorsal layer is considered as the main barrier for drug permeation due to its thickly packed structure with keratin and cells.[11]

In the following project, several active (Iontophoresis, solid microneedles) and passive [nail permeability enhancers (NPEs)] techniques were used to enhance the ungual and trans-ungual delivery of drugs.[12] The application of nail permeation enhancers is the most widely used approach which works by several mechanisms. Some permeation enhancers break the disulfide bonds in the nail plate and some enhance the water holding capacity of nail plate.[12]

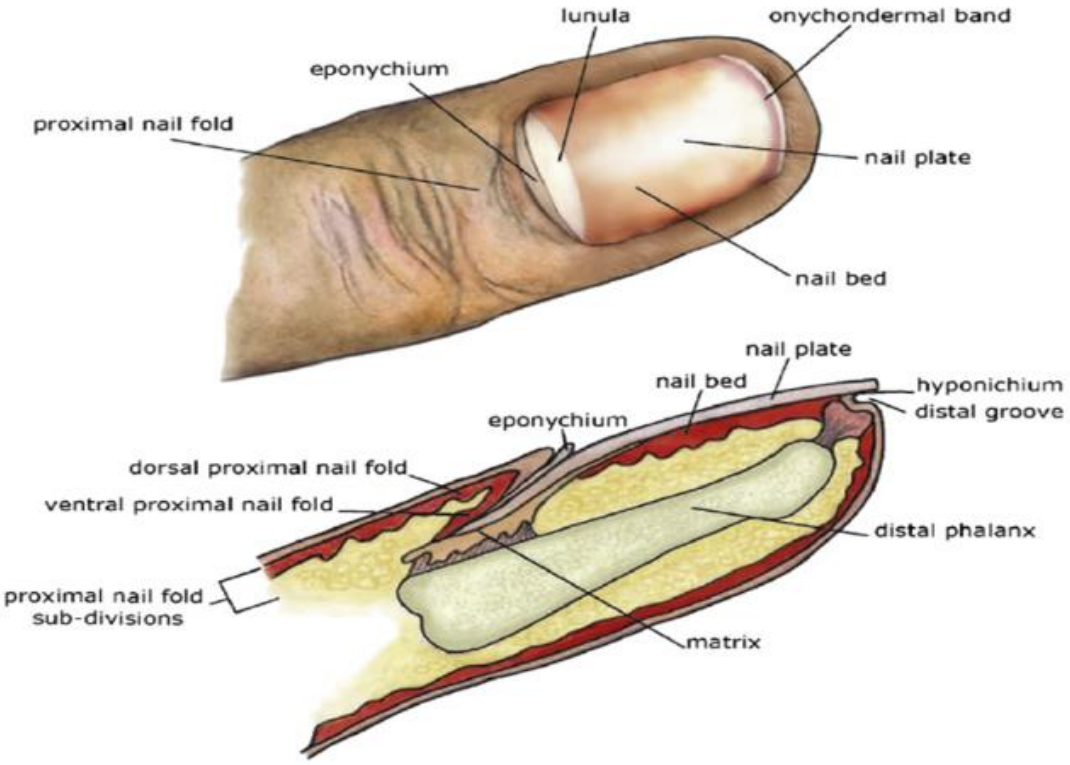


Figure 1. Anatomy of nail apparatus

Iontophoresis is one of the widely studied active technique that enhances the permeation of drugs by the way of passing electrical current across nail plate.[12-16] The amount of drug delivered is directly proportional to the amount of current applied, duration of current application and area

of the nail surface in contact with active electrode. The amount of delivery of drugs can be regulated by adjusting the applied electrical dose (time x current density).[12-16]

Microneedle pre-treatment is novel approach which has already been used to deliver small and large molecules into the skin in a minimally invasive manner.[17-19] The application of microneedles has not been explored before to improve nail delivery of drugs.[18,53] In this project, solid microneedle pre-treatment was investigated as an approach to enhance the delivery of drugs into the nail apparatus.

CHAPTER 2: A NOVEL NAIL LACQUER FORMULATION FOR TRANS-UNGUAL DELIVERY OF APREMILAST

1.0 Introduction

Apremilast is recently approved by FDA as solid oral dosage form for the treatment of skin and nail psoriasis.[5-7] According to clinical studies, an approximately 33.3 % of nail psoriasis patients showed 50% reduction in nail psoriasis severity index (NPSI) when they were administered apremilast tablets twice a day for 16 weeks. However, in case of placebo, 14.9% of patients showed 50% reduction in NPSI.[20] Oral administration of apremilast is associated with severe side effects such as diarrhea, headache, nausea, upper respiratory tract infection, vomiting, runny or stuffy nose or abdominal pain.[20,21]

The current project was designed to investigate the penetrability of apremilast into and across the nail plate, followed by the preparation of a novel nail lacquer formulation to improve its ungual and trans-ungual permeation.

2.0 Materials and methods

Apremilast was purchased from Medkoo Biosciences (Chapel hill, NC). All the surfactants and humectants were gifted by BASF (West Memphis, AR). Cadaver human nails were purchased from Science care (Phoenix, AZ). The solvents were purchased from Fisher Chemicals (Hanover Park, IL). Eudragit® S 100 [Methacrylic Acid - Methyl Methacrylate Copolymer (1:2)] was gifted by Evonik Industries (Germany).

2.1. Preparation of nail lacquer formulation

Nail lacquer formulation was prepared using Eudragit® S 100 (8% w/w) as a film forming polymer. The mixture of ethanol, ethyl acetate and water (63:30:7) were used as a solvent system. In first step, Eudragit® S 100 and apremilast (1 mg/ml) were completely dissolved in ethanol, ethyl acetate and water mixture by continuous shaking on tube rotator for 2 h. Finally, an enhancer was accurately weighed according to table 1 and added to nail lacquer formulation. The enhancers were screened individually by incorporating in nail lacquer formulation.

Name of the enhancer	**% w/w**
Vitamin E TPGS	5
Salicylic acid	5
Dexpanthenol	10
Tocopherol	2
Polyethylene glycol 400	10
Kolliphor™ RH 40	10
Kolliphor® EL	10
Kolliphor™ HS 15	5
Kolliphor® CS 20	5

Table 1. The list of excipients screened for potential permeation enhancement of apremilast.

2.2. Screening of novel permeation enhancers

Human nail plates (0.07cm^2) were used to perform screening studies. Permeation enhancers were screened using TranScreen-N high throughput technique which was mentioned by Nair, et al. Prior to start an experiment, all nail plates were soaked in phosphate buffer saline (PBS) for atleast 2 h. Each nail plate was treated with 100 µl of nail lacquer formulation at room temperature for 24 h. Upon the treatment period, nail plates were washed five times with ethanol and then pat dried with Kimwipes®. Each nail plate was weighed precisely and solubilized in 2 ml of 1 M sodium hydroxide solution by shaking overnight on tube rotator. Hydrochloric acid

(0.4 ml, 5 M) was used to neutralize the alkaline solution, followed by adding 2 ml of ethyl acetate as an extraction solvent. The final mixture was centrifuged at 3000 g for 10 min to separate the solvent layers. Ethyl acetate layer was collected and evaporated using nitrogen gas evaporator to recover the solid crystals of apremilast. Finally, the amount of apremilast was reconstituted in 1 ml of ethanol and quantified using HPLC.[22]

2.3. Characterization of nail lacquer formulation

2.3.1. Stability studies

Stability studies were performed following ICH guidelines recommended for zone II (Mediterranean and subtropical) at 25℃ ± 2 ºC temperature and 60% ± 5% RH (relative humidity) for 3 months.[23] During stability studies, samples of nail lacquer formulation were collected every month. The amount of apremilast contained in the samples was quantified using HPLC method.

2.3.2. Drying time

Approximately 20 µl nail lacquer formulation was applied on human cadaver nail plate and recorded the duration to form a complete dry film.[24]

2.3.3. Differential scanning calorimetry (DSC)

DSC (PerkinElmer, California) was used to investigate the solid state nature of apremilast existed in a film formed by nail lacquer formulation. DSC thermogram was recorded for apremilast, the film formed by nail lacquer formulation and other excipients at heating rate of 30 ^{0}C/min in the range of 30 ^{0}C to 200 ^{0}C. The flow of nitrogen gas was maintained at 22 ml/min.[3]

2.3.4. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy (Cary 660, Agilent Technologies Santa Clara, CA) was performed to evaluate the interactions between excipients and apremilast. FTIR spectra were recorded in the range of 700 - 4000 nm wavelength.

2.3.5. Bioadhesivity of nail lacquer formulation

Bioadhesivity could significantly influence the performance of nail lacquer formulation. Texture Analyzer equipped with a 50-kg load cell (TA XT2i, Technologies Corp, Scarsdale, NY/ Stable Micro Systems, Godalming, Surrey, UK) was used to measure the bioadhesive property. Nail lacquer formulation was applied on a glass slide that was placed on bottom surface of the instrument. Approximately, 0.64 cm^2 human cadaver nail plate was attached with TA-10 probe and dropped it from height of 25 mm at a speed of 1 mm/s with a force of 3.5 N till nail plate touched to the nail lacquer formulation. Nail plate was kept in contact with nail lacquer formulation for 30, 60, or 120 s. The peak force and work of adhesion applied to detach nail plate from nail lacquer formulation were measured and then the results were interpreted using Texture Expert™ software.[24]

2.3.6. Loss on washing studies

The washing studies were performed on human cadaver nail to assess the water resistance property of a film formed by nail lacquer formulation. Prior to start an experiment, all the nails were cleaned with 70% alcohol and pat dried with Kimwipes®. Then, 20 µl of nail lacquer formulation was applied gently on each nail. After complete drying of film, each nail was washed 10 times separately with 1 ml of distilled water. The amount of apremilast contained in washing samples was quantified using HPLC.[24]

2.4 *In vitro* transport studies

Transport studies were performed across human cadaver nail plate using Franz diffusion cells for

7 days. Before starting an experiment, nail plates were soaked in phosphate buffer saline (PBS) for 2 h. Then, they were washed with distilled water and pat dried with Kimwipes®. Each nail plate was secured between donor and receiver compartments with an adapter having 0.3 cm^2 active diffusion area. The top compartment was filled with 100 µl of nail lacquer formulation. The bottom compartment was filled with 20% ethanol. Control was used as a nail lacquer formulation without permeation enhancers. The solution in the bottom compartment was stirred continuously during transport studies using 3 mm size magnetic bar at 600 rpm. Samples were collected from the bottom compartment at certain period of time. The amount of apremilast found in the samples was quantified using HPLC.[13-16]

2.5 Extraction of apremilast from nail plate

After transport studies, an active diffusion area of the nail plate was excised using 0.3 cm^2 metric punch. The nail plates were washed 5 times properly with 70% of ethanol and wiped off with Kimwipes®. Each nail plate was accurately weighed and solubilized in 2 ml of 1 M sodium hydroxide solution by shaking overnight. Hydrochloric acid (5 M, 400 µl) was added to neutralize sodium hydroxide solution. Then, 2 ml ethyl acetate was added to extract apremilast from the suspension. The final mixture was centrifuged at 3000 g for 10 min. Ethyl acetate layer was collected and evaporated using nitrogen gas evaporator to recover the solid crystals of apremilast. Finally, the amount of apremilast extracted from the nail plate was reconstituted in ethanol and quantified using HPLC. The amount of apremilast diffused in peripheral area of the nail plate was extracted following same process which was used for extraction of apremilast from active diffusion area.[13-16]

2.6 Human subject studies

Human ethical committee of Visveswarapura Institute of Pharmaceutical Sciences, Bangalore, India approved the protocol and study design. Six healthy human volunteers in the age range of 25-35 year old participated in human subject studies. The volunteers were divided into two groups: group I was treated with control formulation (nail lacquer formulation without enhancers) and group II was treated with nail lacquer formulation. Prior to application of the formulations, volunteers were asked to clean the finger and thumb nails with 70% alcohol and pat dried with Kimwipes®. Approximately, 20 µl of nail lacquer formulation was applied on each nail plate twice in a day for 15 days. All the volunteers were instructed to avoid cleaning their hands before complete drying of nail lacquer formulation. After 15 days of treatment, distal edge of nail plate was excised and weighed respectively. The amount of apremilast retained in nail plate was extracted using solvent extraction method. Ethyl acetate was used as an extraction solvent. Section 2.5 describes the complete extraction procedure.[24]

2.7. Analytical method

The amount of apremilast was quantified using high performance liquid chromatography system (HPLC, waters, 1525) comprising of an auto sampler (waters 717 plus), Cogent Bidentate C18 analytical column (4.6 mm × 150 mm, Luna, 5.0 µm), waters dual wavelength UV detector (2487). Isocratic HPLC method was performed at 32 ^{0}C using a flow rate of 1.0 ml/min. Apremilast was eluted at 254 nm with mobile phase consisting of acetonitrile and nanopure water (60:40). The pH of the mobile phase was adjusted with 0.1 % of orthophosphoric acid. The HPLC method was validated from the range of 0.01 to 10 µg/ml (R^2 = 0.99).[25]

2.8. Statistical analysis

One-way ANOVA test was performed for statistical analysis of data using SPSS software. The data was considered to be statistical significant as p value found less than 0.05.

3.0 Results and Discussion

Topical therapy is a new emerging therapy for the treatment of nail psoriasis. In recent years, permeation enhancers have been widely used to improve the permeation of drugs across the nail plate. Some enhancers work as a moisturizer (enhance the water holding capacity of the nail plate) and some break the disulfide bonds of keratin in nail plate.[12] In current project, several surfactants, humectants and keratolytic agents were screened to improve the permeation of apremilast into and across the nail plate (Table 1). Eudragit® S 100 was used as a film forming polymer to improve the adhesivity of nail lacquer formulation with nail plate for long period of time. Propylene glycol 400 was used as a moisturizing agent.

High throughput TranScreen-N method was used to screen nail permeation enhancers. As a result of screening studies, dexpanthenol and salicylic acid were found to be the most potential enhancers which were used as the moisturizing and keratolytic agents.[26,27] The result of screening studies is presented as the amount of apremilast retained in nail plate per total weight of nail plate. The amount of apremilast retained in nail plate after application of formulations containing dexpanthenol and salicylic acid was ~2 (13.5±1.2 µg/mg) and ~3 (16.8±2.6 µg/mg) fold more relative to control (5.7±0.9 µg/mg) [$p<0.05$]. Figure 2 illustrates the screening studies data.

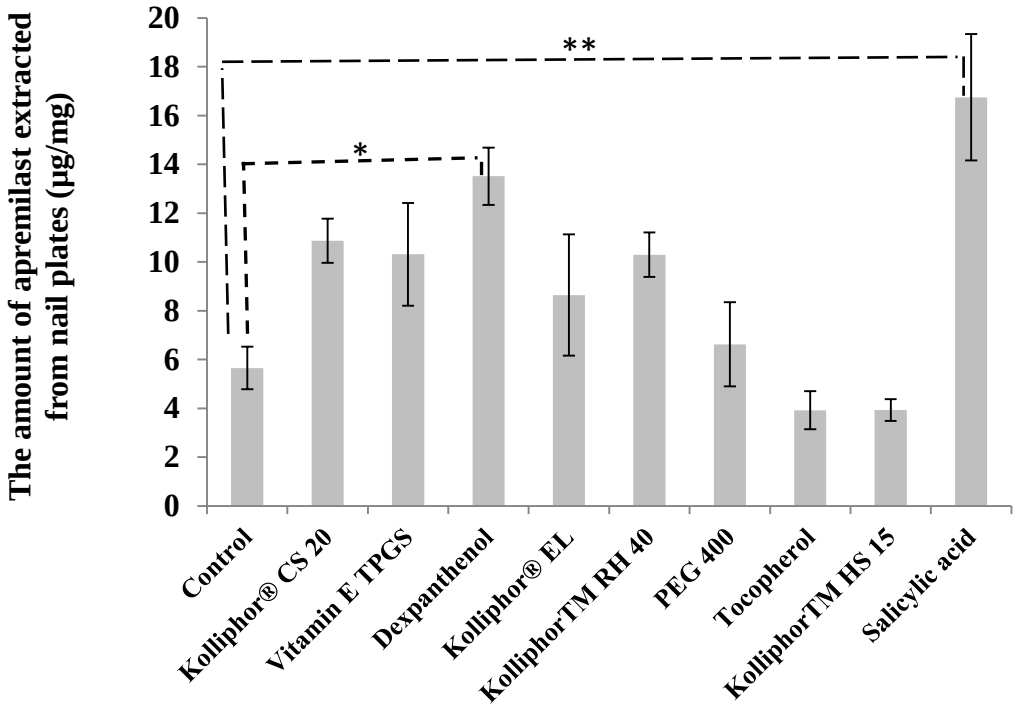


Figure 2. The result of TranScreen-N for identifying the potential enhancers. The data represent mean ± SD of six determinations. *[$p<0.05$], ** [$p<0.05$]

Number	Excipients	% w/w
1	Apremilast	0.1
2	Salicylic acid	5
3	Dexpanthenol	10
4	PEG 400	5
5	Eudragit® S 100	8
6	Water	5
7	Alcohol	45.3
8	Ethyl acetate	21.6

Table 2. The composition of apremilast nail lacquer formulation

Stability studies resulted that nail lacquer formulation was able to stabilize ~ 97.8% of apremilast over 3-month period of time at 25℃/60% RH (Table 3). Drying time studies revealed that nail lacquer formulation was able to form a dry film on human nail plate in ~55 sec.

	% stability of apremilast in the nail lacquer formulation at 25℃/60% RH			
Time	0 month	1 month	2 months	3 months
	100.1±1.15	98.5±1.59	97.08±0.6	97.8±1.02

Table 3. The results of stability studies of apremilast nail lacquer formulation. The data represent mean ± SD of three determinations.

DSC was performed to investigate the solid state nature of apremilast in nail lacquer film. According to DSC thermogram, the pure API of apremilast showed the endothermic peak at 163.8 ºC (Figure 3). However, in case of nail lacquer film, the endothermic peak was absent which indicates the conversion of apremilast into amorphous form or in solid-solid solution.

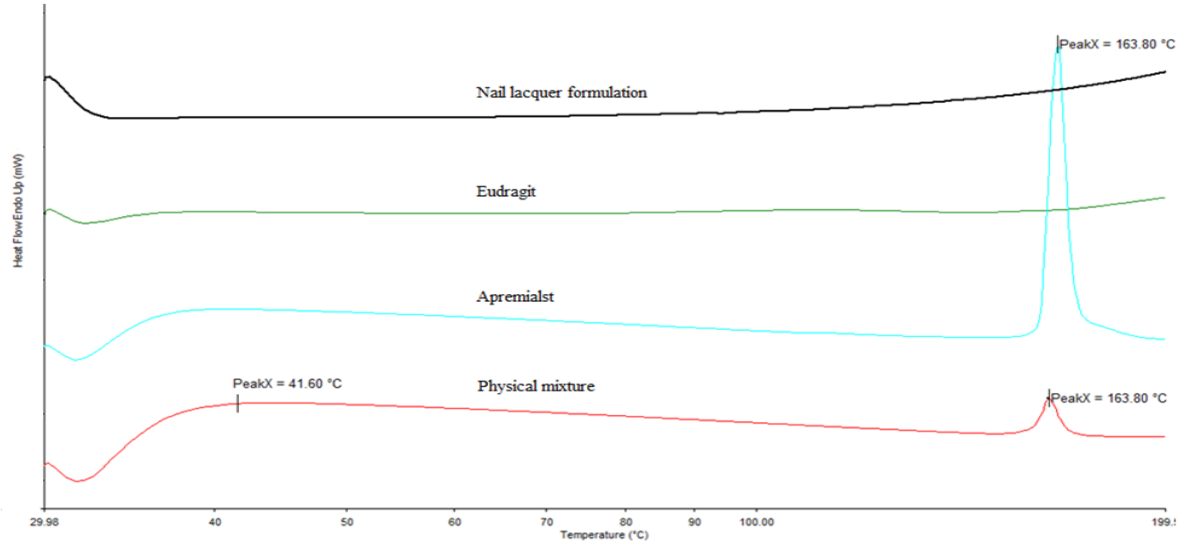


Figure 3.DSC thermogram of apremilast nail lacquer formulation, excipients and physical mixture of apremilast with excipients.

FTIR analysis was performed to investigate the formation of new hydrogen bonds between apremilast and Eudragit® S 100 polymer in nail lacquer film. FTIR spectra were recorded for the apremilast, Eudragit® S100 and a nail lacquer film with permeation enhancers. . As a presence of permeation enhancers may affect the proper understanding of drug-polymer interaction, the nail lacquer film was prepared devoid of penetration enhancers.

The structure of apremilast illustrates that it can potentially act as either proton acceptor (amide and ketone carbonyl groups, -C=O) or proton donor (amide functional group, -NH-$COCH_3$) (figure 3). The stretching vibration of amide –C=O, ketone –C=O, and amide –N-H groups of apremilast was found at 1687 cm^{-1}, 1763 cm^{-1} and 3362 cm^{-1} (figure 4). The structure of Eudragit® S 100 polymer has one proton acceptor (carbonyl, C=O) and one proton donor (hydroxyl, -OH) which showed the stretching vibration at 1724 cm^{-1} (carbonyl group) and 3445 cm^{-1} (hydroxyl group) (figure 4).

FTIR spectra of nail lacquer illustrated the shift in the stretching vibration of carbonyl and amide –N-H groups of apremilast which were recorded at 1699 cm^{-1}, 1781 cm^{-1} and 3399 cm^{-1}. According to previous literature, the shift in the stretching vibrations indicates the formation of new hydrogen bonds between drug and polymer. In the case of nail lacquer, the peaks at 1699 cm^{-1} and 1781 cm^{-1} were attributed to apremilast carbonyl groups which formed H-bonds with the polymer, whereas a new peak at 3399 cm^{-1} was characterized to apremilast amide –N-H group that formed H-bond with the hydroxyl group of the polymer (Figure 4).

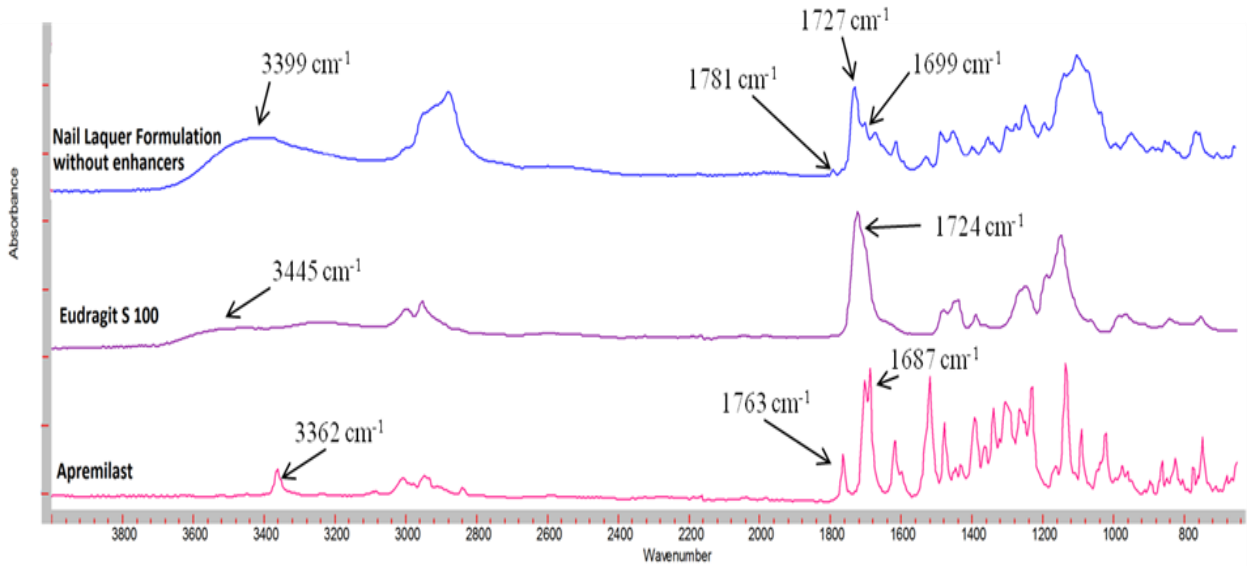


Figure 4. FTIR Spectra of apremilast nail lacquer formulation and excipients.

The Peak force and work of adhesion were found to increase with enhancement of contact time between the nail plate and nail lacquer formulations (placebo and apremilast load) (Figure 5, 6). The peak force and work of adhesion at any contact time period were higher for the placebo lacquer compared to apremilast-loaded lacquer, while there was no significant difference found between each other ($p > 0.05$). To conclude, apremilast-loaded nail lacquer film was found to have sufficient adhesiveness to be retained on the surface of nail plate for prolonged period of time. In addition, its adhesivity was not changed significantly after adding drug and other excipients compared to placebo.

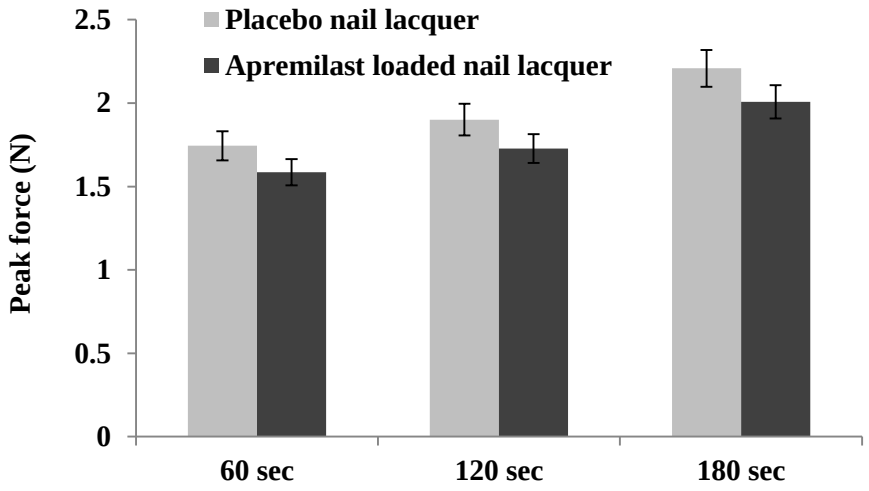


Figure 5. Peak adhesive force (N) of apremilast loaded nail lacquer formulation and placebo. The data represent mean ± SD of three determinations. ($p>0.05$)

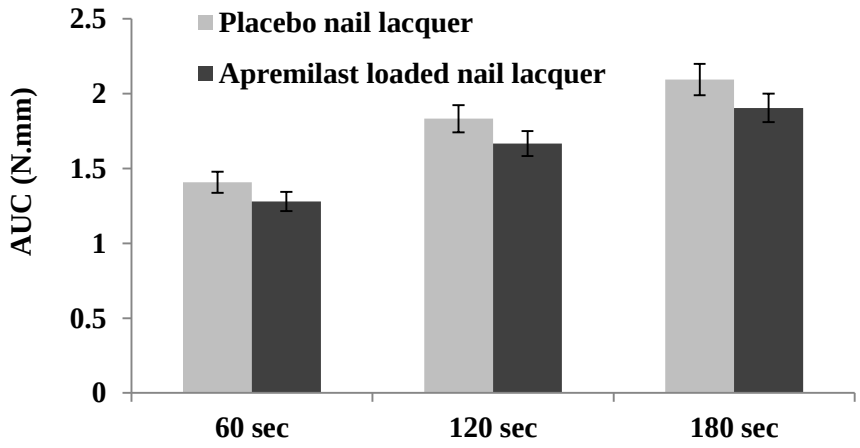


Figure 6. Adhesion of apremilast loaded nail lacquer formulation and placebo. The data represent mean ± SD of three determinations. ($p>0.05$)

Loss on washing (LOW) studies was performed on human cadaver nails. The amount of apremilast lost after 10^{th} washing was approximately ~5% of the total amount contained in nail lacquer formulation (Figure 7).

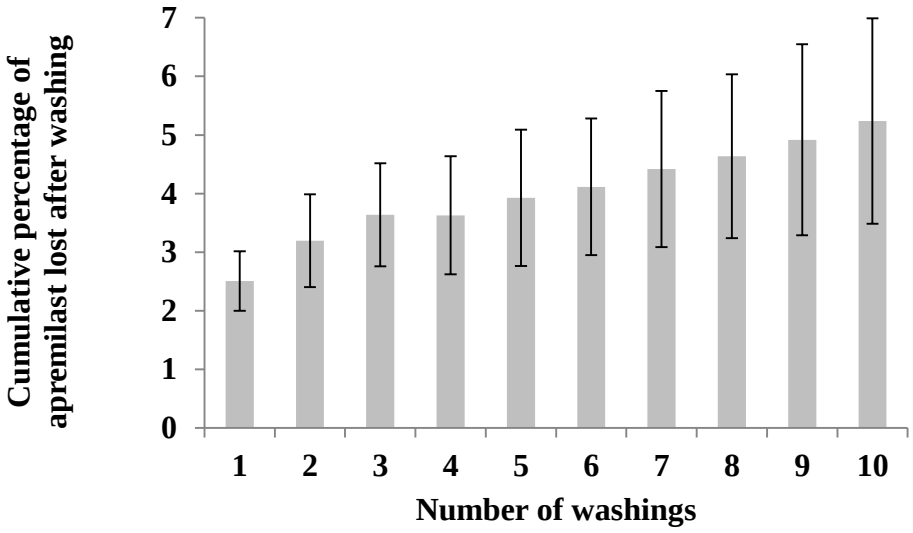


Figure 7. The cumulative amount of apremilast lost after 10 times washing a film formed due to application of nail lacquer formulation. The data represent mean ± SD of three determinations.

After 7 days of *In vitro* permeation studies, the amount of apremilast found in the receiver compartment in case of nail lacquer formulation was 0.52±0.07 µg/cm^2 which was ~3 fold more when compared with control (0.19±0.02 µg/cm^2) (Figure 8). The amount of apremilast retained in the nail plate after transport studies in case of nail lacquer formulation (1.26±0.18 µg/mg) was ~2 fold more compared to control (0.57±0.07µg/mg) (Figure 9). The amount of apremilast diffused in the peripheral area was 0.39±0.09 µg/mg which was ~2 fold more compared to control (0.23±0.06 µg/mg) (Figure 10). According to a result of *in vitro* studies, nail lacquer formulation was able to deliver significant amount of apremilast into and across the nail plate related to control ($p<0.05$).

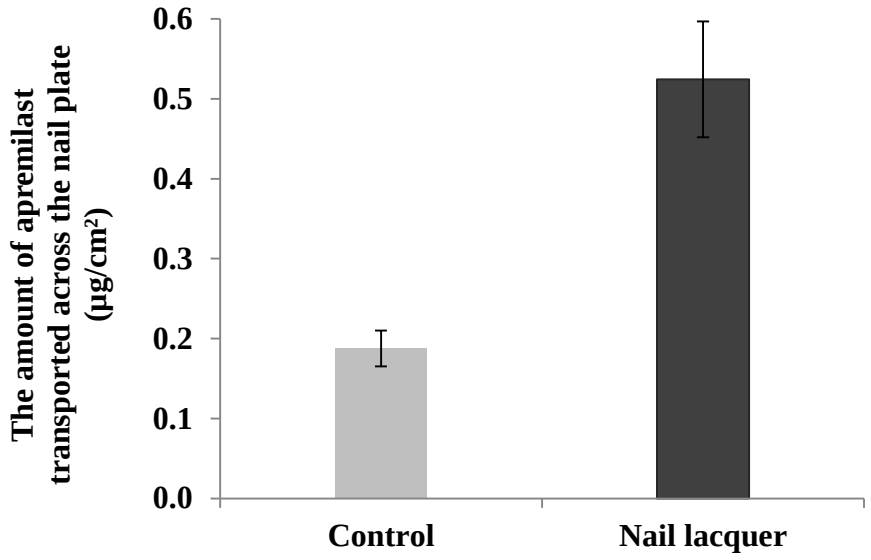


Figure 8: The amount of apremilast permeated across the nail plate after 7 days of *in vitro* permeation studies. Control was used as a nail lacquer without permeation enhancers. The data represent mean ± SD of six determinations.

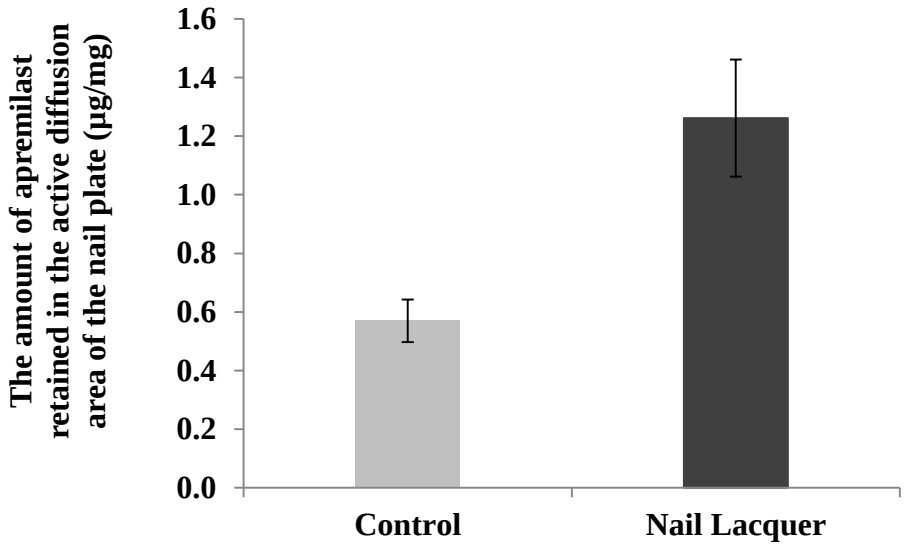


Figure 9: The amount of apremilast retained in active diffusion area of the nail plate after 7 days *in vitro* of permeation studies. Control was used as a nail lacquer without permeation enhancers. The data represent mean ± SD of six determinations.

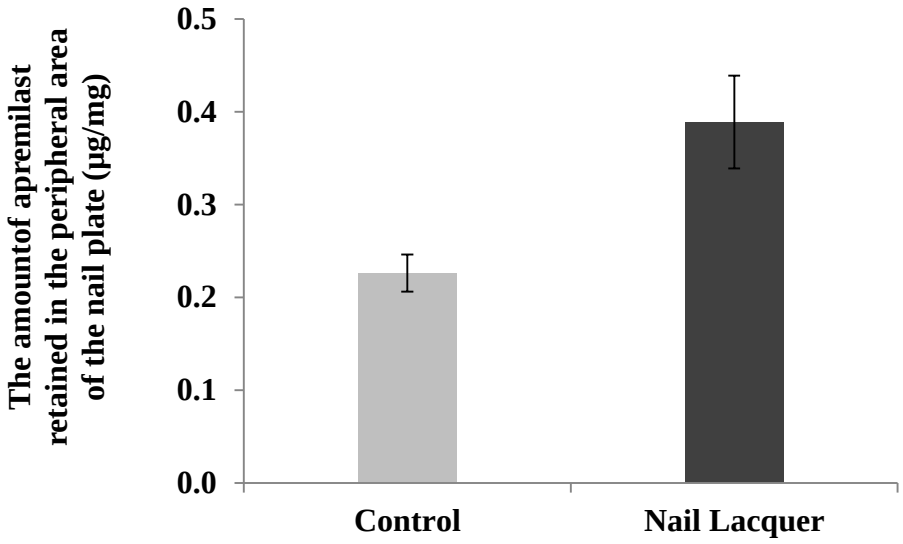


Figure 10: The amount of apremilast retained in the peripheral area of the nail plate after 7 days of *in vitro* permeation studies. Control was used as a nail lacquer without permeation enhancers. The data represent mean ± SD of six determinations.

Human subject studies were performed to verify the results of *in vitro* studies. Six human volunteers participated in the studies who were in the age range of 25-35 year old. The amount of apremilast retained in nail plate after 15 days application of nail lacquer formulation was 0.93±0.14 µg/mg, whereas it was 0.41±0.04 µg/mg in case of control. Clinical studies concluded that nail lacquer formulation was able to improve the loading of apremilast in nail plate ~2 fold more compared to control.

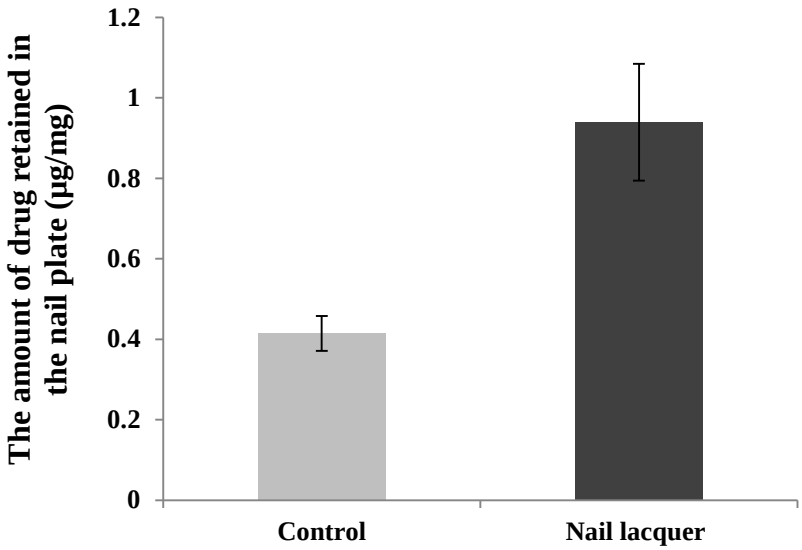


Figure 11: The amount of apremilast retained in nail plate of human volunteers. The volunteers were divided in two groups, each group had 3 volunteers. Nail lacquer formulation was applied on two nails of each hand (thumb and index finger) of group I volunteers. Control (nail lacquer without enhancers) was applied on two nails of each hand (thumb and index finger) of group II volunteers.

4.0 Conclusions

Nail lacquer formulation showed the ability to form a water resistant film on the nail plate which retained the drug on nail plate for prolonged duration and facilitated drug penetration into the nail plate. Permeation enhancers were able to render the formulation relatively more efficient in enhancing the amount of apremilast delivered into and across the nail plate from the topical nail lacquer formulation.

CHAPTER 3: TRANS-UNGUAL DELIVERY OF ITRACONAZOLE HYDROCHLORIDE BY IONTOPHORESIS

1. Introduction

Itraconazole (ITR) is a triazole antifungal drug for the treatment of onychomycosis.[12] However, long time oral use of ITR is known to cause severe gastric and systemic side effects such as nausea and vomiting.[3] According to the FDA, "patients who have signs of ventricular dysfunction such as congestive heart failure or a history of congestive heart failure should not take ITR orally for treatment of onychomycosis".[33]

Passive trans-ungual delivery of drugs is limited due to hard nature of the nail plate.[1] In current project, iontophoresis technique was used to deliver ITR across the nail plate. ITR-HCl salt was prepared to render it amenable to iontophoresis. In previous literatures, iontophoresis has been reported as a successful technique to deliver the significant of drug into the nail apparatus.[13-16,30]

2. Methods and materials

2.1 Materials

Itraconazole (ITR, molecular weight = 705.2) was purchased from VWR International (Atlanta, GA). Phosphate buffer saline at pH 7.4 (0.138 M of sodium chloride, 0.0027 M potassium chloride) and acetonitrile were purchased from Omnipur and Fisher Chemicals (Hanover Park, IL). Excised human cadaver toes were purchased from Science Care (Phoenix, AZ). The Porcine hoof was obtained from Pontotoc slaughtering house (Mississippi, USA).

2.2 Synthesis of ITR-HCl

ITR suspension (40 g/800 mL of acetone) was prepared in a round bottom flask with three necks. Anhydrous hydrogen chloride gas was bubbled slowly into the suspension while heated under reflux. Hydrogen chloride gas was prepared using solid sodium chloride and concentrated sulfuric acid (H_2SO_4). After 30 min continuous heating, the suspension turned into solution and eventually in the next 5-10 min, ITR-HCl was precipitated. The hydrogen chloride gas was continued to pass for next 2 h. The slurry was allowed to stand for 24 h and then the precipitate was collected, washed with acetone and dried at 40^0 C.[34]

2.3 Characterization of ITR-HCl

2.3.1 Differential scanning calorimetry (DSC)

The DSC (PerkinElmer, California) for ITR and ITR-HCl was recorded in the range of 30 ^{0}C to 250 ^{0}Cat a heating rate 10 ^{0}C /min and nitrogen flow rate of 22 ml/min.

2.3.2 Mass spectroscopy

Mass spectroscopy of ITR and ITR-HCl was performed using matrix-assisted laser desorption/ionization technique (MALDI- SYNAPT MS/HDMS). Dimethyl sulfoxide was used to dissolve the samples. Samples were scanned from intensity of 200 m/z to 5000 m/z.

2.4 Solubility measurement

Solubility studies of ITR-HCl was carried out in water, isopropanol and mixtures of water and isopropanol (90:10, 80:20, 70:30, 60:40 and 50:50 v/v percentage) at pH 3 for preparing appropriate solution to perform iontophoresis. The pH of the solvent system was adjusted using 0.01 N HCl. Excess amount of ITR-HCl was added to the solvents and sonicated for 15 min at room temperature. Suspensions were shaken continuously on the rotary shaker for 2 days, and then filtered using disposable syringe filters (0.45 μm). Amount of dissolved ITR was estimated

by HPLC.

2.5 Antifungal activity testing of ITR and ITR-HCl

Clinical and laboratory standards institute method was used for antifungal activity testing of ITR and ITR-HCl. Testing Organisms were obtained from the American Type Culture Collection (Manassas, VA) (*Candida albicans*, *Candida krusei*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Trichophyton Rubrum*). The suspensions of testing organisms for *Candida* and *Cryptococcus* species were prepared from 3-5 colonies using sabouraud dextrose (SD) agar in sterile normal saline and incubated for 24 -72 h. In the case of *trichophyton* species, potato dextrose medium was used to prepare the suspension and allowed to incubate for 1 to 2 weeks. Standard curves between optical density and CFU/ml of saline suspensions of test organisms were prepared to calculate the sample assay inocula at 630 nm.

Assay inocula were prepared by diluting organism suspension in the incubation broth (RPMI 1640 broth for *Candida* spp., SD broth for *C. neoformans*, and 5% Alamar blue–RPMI 1640 broth for *Trichophyton* spp) to reach the final inocula (1.5×10^3 CFU/ml for *Candida* spp. and *Cryptococcus* spp. or 2.7×10^4 CFU/ml for *Trichophyton* spp). Verification of inocula for each assay was carried out by platting on SD or PD agar for colony reckoning.

ITR and ITR-HCl samples were diluted by 20% dimethyl sulfoxide-saline to provide enough volume for all organisms and transferred in duplicate to 96 well flat-bottom microplates. ITR and ITR-HCl samples were assayed using two folds serial dilutions by preparing a total of 11 test concentrations. 10 µL of all diluted samples were transferred in duplicate microplate as in template and made up the final volume up to 200 µl of all diluted samples using organism inocula.[35]

Reading of *Candida* spp. and *C. neoformans* in the duplicate microplates were done using the Biotek Power wave XS plate reader at 530 nm after and before incubation (*Candida* spp. at 35 °C for 48 h; *C. neoformans* at 35 °C for 72 h). Reading of A. *fumigatus* and *Trichophyton* spp were carried out using the Polarstar Galaxy plate reader (BMG Lab Technologies, Germany) at the excitation and emission wavelength of 544 nm and 590 nm prior to and after incubation (*Trichophyton* spp. at 35 °C for 5 days). IC_{50}, MIC and MFC were calculated using the XLFit software (dose-response model 201; IDBS, Alameda, CA).[35]

The *Minimum inhibitory concentration* (MIC) is defined as the lowest concentration that allows no detectable growth (or no more than 20% growth for the azoles and caspofungin). The Minimum fungicidal concentration (MFC) is defined as the lowest test concentration that allows no growth of the organism on agar. Half-maximal inhibitory concentration (IC_{50}) is defined as a quantitative measurement of amount of drug that is needed to inhibit half of the biological process.[35]

2.6 *In vitro* drug transport studies across hoof membrane

In vitro drug transport studies were performed across the porcine hoof membrane as a model for human nail using Franz diffusion cells. Hoof membrane was excised, cleaned and prepared to have a thickness of 150-200 μm using scalpel. The hoof membrane was hydrated with phosphate buffer saline overnight prior to securing it between donor and receiver compartments. Active diffusion area (0.3 cm^2) of the hoof membrane used to perform transport studies. For iontophoresis, platinum and silver chloride electrodes were fixed at 2 mm distance from the hoof membrane in the donor and receiver compartments respectively. Constant DC current (0.5 mA/cm^2) was applied using custom designed iontophoresis device (Active dose II iontophoresis delivery unit, Transport Pharmaceuticals, Boston, MA). The donor compartment was filled with

0.5 ml of ITR-HCl solution prepared by dissolving 1 mg of drug in 1 mL of water and isopropanol (1:1) mixture at pH 3. Receiver compartment was filled with 5 ml mixture of isopropanol and phosphate buffer saline (1:1) mixture at pH 3. 3 mm magnetic bars were used to stir (600 rpm) the solution in receiver compartment for uniform distribution of the drug. Passive transport studies were performed simultaneously using same set up of Franz diffusion cells without application of current. Samples (200 µl) were collected at fixed time points from the receiver compartment and analyzed by HPLC.[3,16]

The passive and iontophoresis transport studies were performed following two different protocols. First protocol included continuous studies for 24 h and Pulsed protocol involved transport studies for 8 h/day for three days. In case of continuous protocol studies, the formulation in the donor was replaced every 8 hours with fresh formulation. In the pulsed mode, the hoof surface was washed after each 8-hour episode of transport studies and allowed to be in contact with the receiver compartment for remaining 16 hours.

2.7 Extraction of ITR from hoof membrane

After *in vitro* transport studies, the hoof membrane was detached from the adapter. Active diffusion area (0.3 cm^2) of the membrane was marked and excised using metric punch apparatus before washing. Each nail plate was washed out by shaking (2 times) in the water and isopropanol mixture (1:1 at pH 3) and 95% ethanol with the help of forceps. This process was performed alternatively five times with both solvents. The nail plate was wiped each time using Kimwipe®. Each plate was cut into small pieces and solubilized in 2 ml of 1 M sodium hydroxide solution by subjecting to ultrasonic treatment at 37 ^{0}C for 2 h. 6 ml of ethyl acetate was added to the final solution and ethyl acetate layer was separated out to another glass vial. 2 ml of 1 M hydrochloric acid was added to the remaining sodium hydroxide layer for reverse

extraction. To this solution, another 6 ml of ethyl acetate was added and ethyl acetate layer was separated out to another glass vial. Both ethyl acetate glass vials were mixed together and ethyl acetate was evaporated using nitrogen gas to get solid crystals of ITR-HCl. Finally, ITR-HCl was dissolved in water and isopropanol mixture (1:1) at pH 3 prior to analysis.[36]

2.8 Iontophoresis with excised human cadaver toe

Hydroxypropylmethyl cellulose gel (2% HPMC) incorporated with ITR-HCl was prepared using water and isopropanol (1:1) mixture adjusted to pH 3. Excised human cadaver toe model was used to perform the *ex vivo* drug transport studies. The cadaver toes were dipped in 0.5 % gentamycin solution and dried one day prior to use in the study and stored at -20 °C, the toes were thawed at room temperature and then the studies were performed at room temperature conditions. Custom-designed patch was prepared to carry out the transport studies similar to that discussed earlier[16]. Adhesive backing membrane was used to fix electrode to the nail plate. Polyurethane foam pad was used to expose the drug and current on the fixed area. HPMC gel was filled up in the fixed area using spatula. Counter electrode filled with conductive gel (no drug) was adhered to the bottom of the toe. Anodal and cathodal electrodes were used as the active and counter electrodes respectively. A constant DC (0.5 mA/cm^2) was applied for 24 h (again following two different protocols as described in section 2.6) using iontophoresis device. Passive transport studies were performed simultaneously with iontophoresis studies using same set up on the toe without current application. The amount of drug was estimated by HPLC after extraction of drug from the nail plate and nail bed.[16,37]

2.9 Extraction of ITR from nail plate and nail bed

Following transport studies, the nail plate was detached from the intact toe using blunt forceps and scalpel. Nail surface was washed (protocol discussed in section 2.7) to get rid of the adhering

drug. Active diffusion area of nail plate was excised using a metric punch. Eventually, amount of ITR was extracted from the nail plate and measured.

Nail bed was separated carefully from the intact toe. Nail bed was homogenized and dissolved in the 1 M sodium hydroxide. The drug was extracted from sodium hydroxide solution using same procedure, detailed in section 2.7.[36]

2.10 Analytical method

The amount of ITR was determined by high performance liquid chromatography system (HPLC, waters, 1525) consisting of an auto sampler (waters 717 plus), phenomenex C18 (2) 100 R analytical column (4.6 mm × 150 mm, luna, 5.0 µm), waters dual wavelength UV detector (2487). Mobile phase was prepared by combination of three solvents, Acetonitrile, nanopure water and diethylamine (70:30:0.05). Elution of drug was carried out isocratically at 32 ^{0}C using a flow rate of 1.0 ml/min and 30 µl injection volume. ITR was detected at 261 nm. Calibration curve was prepared using a range from 0.01- 10 µg/ml (R^2 = 0.99).[33]

2.11 Statistical analysis

Statistical analysis of ITR-HCl permeation studies was performed by student t-test using SPSS software. The P value less than 0.05 was considered significant difference.

3. Results and Discussion

ITR-HCl was characterized by Differential scanning calorimetry (DSC) and MASS spectroscopy. According to the DSC thermogram, sharp melting endothermic peak was found in case of ITR at 171 ^{0}C (Figure 17). However, endothermic melting peak was not found in case of ITR-HCl indicating likely modification of the base into salt.[33]

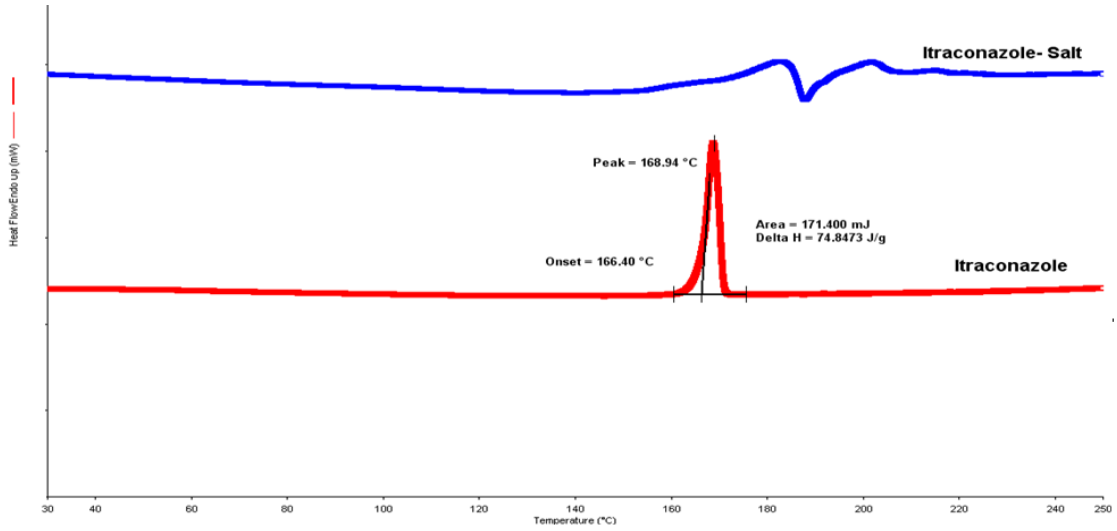


Figure 12. DSC (PerkinElmer, California) thermogram of ITR and ITR –HCl

Mass Spectroscopy of ITR and ITR-HCl was carried out by Matrix-assisted laser desorption/ionization technique (MALDI- SYNAPT MS/HDMS). According to mass spectra, peak of ITR was found at 705.64 m/z. ITR-HCl formation was confirmed by appearance of peak at ~ 741.02 m/z.

Solubility studies of ITR-HCl were performed in water, isopropanol, and mixture of water and isopropanol at pH 3 (Table 5). The maximum solubility of ITR-HCl found in water and isopropanol mixture (50:50 v/v) at pH 3 was 37.52 mg/ml which was ~ 181 folds more when compared with ITR (0.207 mg/ml) (Table 5).

	Solubility of ITR and ITR-HCl (mg/ml)							
	Water	Isopropanol	Water pH 3	Water: Isopropanol ratio (pH 3)				
				90:10	80:20	70:30	60:40	50:50
ITR	--	0.288	--	--	--	--	0.112	0.207
ITR-HCl	0.01	15.15	0.04	0.06	0.09	0.13	0.41	37.52

Table 4. The solubility of ITR and ITR-HCl (mg/ml) in different solvent systems

Antifungal activity assays of ITR and ITR-HCl were performed on the fungal cultures such as *T. rubrum, C. albicans, C. krusei, A. fumigatus and C. neoformans*. According to the results, antifungal activity of ITR and ITR-HCl did not differ significantly ($p>0.05$) in terms of IC50, MIC and MFC levels against all the fungal species tested in this work (Table 6).

	ITR (μg/ml)			ITR-HCl (μg/ml)		
Organisms	IC50	MIC	MFC	IC50	MIC	MFC
T. rubrum	0.063 ± 0.02	0.26 ± 0.08	>20.0	0.06 ± 0.01	0.31 ± 0.05	>20.0
C. albicans	0.18 ± 0.07	>20.0	>20.0	0.11 ± 0.05	>20.0	>20.0
C. Krusei	0.23 ± 0.03	0.87 ± 0.001	1.15 ± 0.25	0.27 ± 0.144	0.84 ± 0.005	1.04 ± 0.15
A. fumigatus	0.50 ± 0.17	1.04 ± 0.35	6.33 ± 1.35	0.29 ± 0.03	1.06 ± 0.25	6.67 ± 1.04
C. neoformans	<0.02	0.027 ± 0.002	0.03 ± 0.001	<0.02	0.02 ± 0.002	0.03 ± 0.001

Table 5. Antifungal activity of ITR and ITR-HCl on different fungal species. The data represent mean ± SD of three determinations. ($p>0.05$)

***In vitro* ITR-HCl Transport studies**

Transport studies of ITR-HCl were performed using porcine hoof as well as excised human toe model. The transport studies were performed by passive and iontophoresis modes using two different protocols. Continuous protocol involved continuous application of formulation for 24 hours. However, in case of continuous protocol also, the applied formulation was replaced every 8 hours for the sake of having sufficient chloride ions in the donor compartment. In non-continuous or pulsed protocol, the duration of application was at 8 h per day for three days (equivalent to 24 hours) across the porcine hoof membrane as well as in human toe model. These

protocols were selected to compare the drug delivery efficiency in two different application conditions. The continuous protocol represents a condition where the subject would be applied with a device for prolonged duration. The pulsed protocol represents wearing the device only for a few hours a day for multiple days. In case of *in vitro* studies using Franz cell, pH 3 solvent system was used in the receiver compartment to maintain sink conditions. Previous studies have shown that formulation with pH 3 did not affect the constitution or permeability of the nail plate.[2,8]

Anodal iontophoresis was performed because of positive charge on the ITR. Platinum wire was used as an anodal electrode in the donor compartment because ITR was found to be precipitated due to pH change (pH 3 to ~3.8) of drug solution in the donor compartment by interaction with Ag electrode. In case of platinum wire, pH of the donor compartment was dropped from 3.0 to 2.5 after the application of iontophoresis for 8 h. After every 8 h, drug solution in the donor compartment was replaced with fresh drug solution to avoid further drop in pH. AgCl electrode was used as cathode electrode in the receiver compartment. The receiver compartment pH was increased to 3.4 at the end of 24 hours. Replacement of fresh buffer solution after each sampling was found to keep the pH from going above 3.4.

Passive versus iontophoresis

In case of continuous protocol, the cumulative amount of ITR in the receiver compartment after application of iontophoresis across the hoof membrane was 0.91 ± 0.11 μg/cm² which was ~ 30 folds ($P<0.05$) more than passive (0.03 ± 0.01 μg/cm²). The amount of drug retained in the hoof membrane by iontophoresis was 4.8 ± 1.2 μg/mg which was ~ 5 folds ($P<0.05$) more than passive (0.95 ± 0.54 μg/mg) (Table 7).

Amount of ITR				
Mode of drug loading	*In vitro* studies			
	24 h study		3 days (8 h/day) study	
	Receiver compartment (µg/cm²)	Hoof membrane (µg/mg)	Receiver compartment (µg/cm²)	Hoof membrane (µg/mg)
Passive	0.03 ± 0.01	0.95 ± 0.54	0.08 ± 0.01	1.3 ± 0.60
Iontophoresis	0.91 ± 0.11	4.8 ± 1.2	2.12 ± 0.30	4.95 ± 1.52

Table 6. In case of *in vitro* transport studies, the amount of ITR was found in the receiver compartment (µg/cm²) and hoof membrane (µg/mg) after continuous and pulse applications of iontophoresis for 24 h and 3 days (8 h/day). The data represent mean ± SD of six determinations.

In case of pulsed protocol, the cumulative amount of drug transported in the receiver compartment by iontophoresis was 2.12 ± 0.30 µg/cm² which was ~ 27 folds ($P<0.05$) more when compared to the passive (0.08 ± 0.01 µg/cm²). On the other hand, the amount of drug found in the hoof membrane by the application of iontophoresis was 4.95 ± 1.52 µg/mg which was ~ 4 folds ($P<0.05$) more than passive (1.3 ± 0.60 µg/mg) (Table 7). These studies have clearly demonstrated the ability of iontophoresis to enhance the delivery of ionic drugs across the nail plate. Iontophoresis was also found to enhance the drug holding capacity of the nail plate.

Amount of ITR				
Mode of drug loading	*Ex vivo* studies			
	24 h study		3 days (8 h/day) study	
	Nail bed (µg/mg)	Nail plate (µg/mg)	Nail bed (µg/mg)	Nail plate (µg/mg)
Passive	0.003 ± 0.09	1.61 ± 0.73	0.11 ± 0.07	1.75 ± 0.98
Iontophoresis	1.17 ± 0.60	4.64 ± 1.14	2.65 ± 1.09	4.96 ± 1.64

Table 7. In case of *ex vivo* transport studies: the amount of drug was found in the nail bed

(μg/mg) and nail plate (μg/mg) after continuous and pulse applications of iontophoresis for 24 h and 3 days (8 h/day). The data represent mean ± SD of six determinations.

Continuous versus pulsed protocol

In both passive as well as iontophoresis modes, the permeation of drug across the hoof membrane was significantly higher in case of pulse protocol as compared to continuous protocol. In the case of pulse protocol, although the duration of application of formulation is same as continuous protocol, there is pause time between the episodes, during which significant amount of drug could diffuse into the sub-ungual tissues (receiver compartment in case of Franz cell studies). This is likely to render the nail more receptive to drug uptake during the subsequent episode of application. However, in case of continuous protocol, the saturation of nail plate is likely to hamper the delivery of drug. However, regardless of the protocol, the amount of drug in the hoof membrane appears to saturate and did not differ significantly between continuous and pulsed protocols.

Human nail Vs porcine hoof model

Porcine hoof has been suggested as a good model for human nail plate.[38] A good correlation between the permeability of drugs across the bovine hoof with that across the human nail plate has been reported by Mertin and Lippold.[39] To assess if there exists any correlation between the porcine hoof in Franz cell model with excised cadaver toe model, two correlation plots were created. The amount of drug permeated across the hoof membrane at a given mode and protocol of delivery was matched with the amount of drug permeated across the nail plate into the nail bed when same delivery mode and protocol was used. Similarly, the drug loaded in the hoof in Franz cell experiments was matched with the levels in the nail plate in toe model. The drug load

in the porcine hoof membrane vs drug loaded in the nail plate showed an excellent correlation (R^2=0.93) (Figure 18). Whereas, the correlation between the amount of drug permeated across the hoof membrane into the receiver compartment and the amount of drug found in the nail bed was relatively modest (R^2 =0.56) (Figure 19). The reason for this poor correlation is likely due to lack of clearance in the toe model. Although, the few number of data points are available for correlation, there appears to be a clear trend of positive correlation which is likely to strengthen with the inclusion of additional data in the future. The present studies have demonstrated that the excised human toe model could be an acceptable model to investigate the ungual drug delivery, despite its limitations.

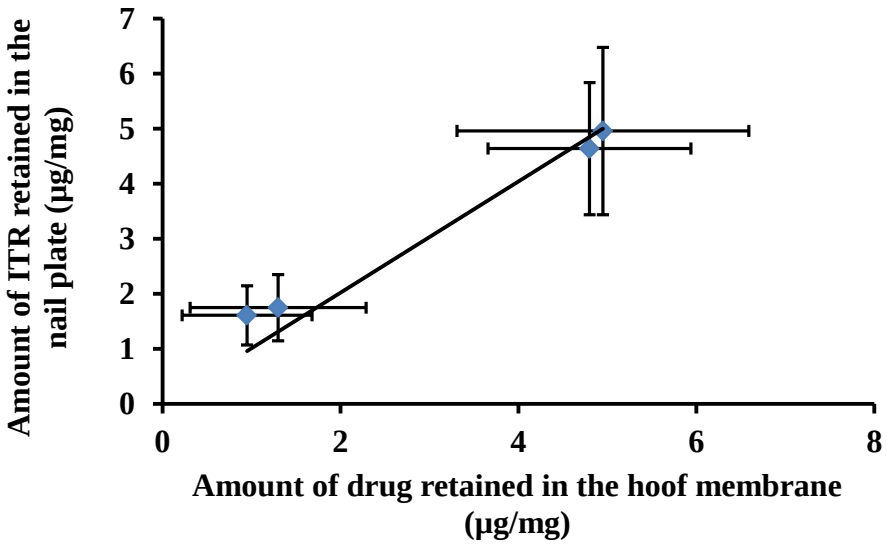


Figure 13. Correlation between amount of drug retained in the nail plate and amount of drug retained in the hoof membrane after transport studies

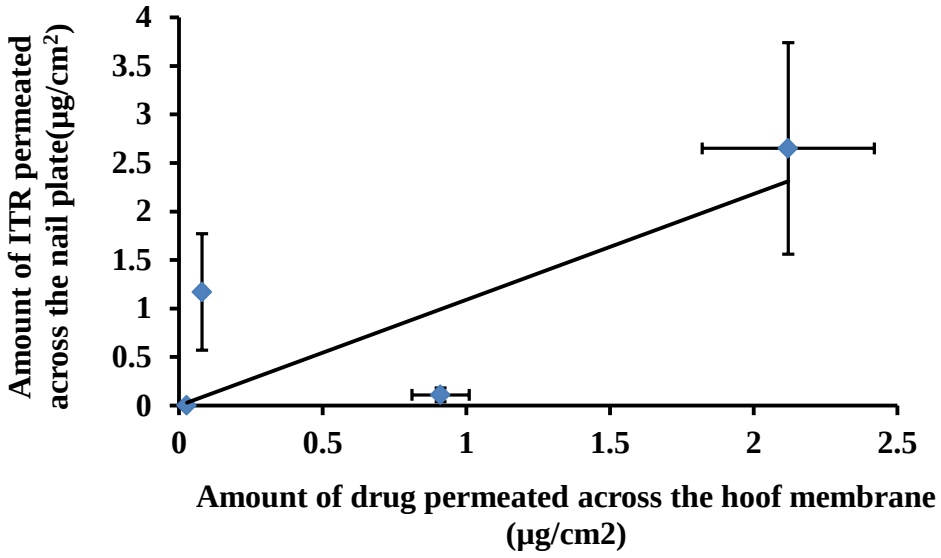


Figure 14. Correlation between amount of drug permeated across the nail plate and amount of drug permeated across the hoof membrane.

4. Conclusion

In vitro and *ex vivo* transport studies have demonstrated the feasibility of iontophoresis technique to enhance the trans-ungual delivery of ITR. Iontophoresis also enhanced the amount of drug loaded in the nail/hoof. Pulsed application protocol was found to be superior over the continuous application protocol in both passive as well as iontophoresis mode of trans-ungual drug delivery. This means in clinical practice, dividing the duration of application into multiple episodes would be more beneficial to the subject than continuous application of iontophoresis over long time.

CHAPTER 4: IONTOPHORESIS FOR DRUG DELIVERY INTO THE NAIL APPARATUS: EXPLORING HYPONYCHIUM AS THE SITE OF DELIVERY

1. Introduction

The topical delivery of antifungal drugs from dorsal side of the nail plate to deeper layers and nail bed is limited and tedious due to poor permeability of the nail plate.[2,3,11] Therefore, the current project was aimed to investigate the feasibility of utilizing the hyponychium pathway to deliver antifungal drugs directly into the ventral layer of the nail plate and other parts of the nail apparatus.

Naturally, nail plate has a structure that allows the distribution of drug molecule from one region to other regions of the nail plate by lateral diffusion.[40] However, passive lateral diffusion of drug in nail plate is very tedious.[40] Therefore, iontophoresis technique was used as a tool to improve the delivery and distribution of the drug into the ventral layer of the nail plate. Iontophoresis delivery has already been reported to enhance the delivery of drugs from dorsal to ventral layer of the nail plate.[3,8,13,41]

In vitro transport studies were performed across the human cadaver nail plates. Sodium fluorescein was used as a maker to investigate the feasibility of application of iontophoresis to propagate the substrate in the ventral layer of the nail plate. Further, *ex vivo* investigations were performed on excised human cadaver toe using terbinafine hydrochloride, as a leading antifungal model drug.

2. Materials and methods

2.1. Materials

Terbinafine hydrochloride and sodium fluorescein were purchased from Sigma-Aldrich (St. Louis, MO). Human nails and excised human cadaver toes were purchased from Science care (Phoenix, AZ). Phosphate-buffered saline (PBS) and acetonitrile were purchased from Omnipur and Fisher chemicals (Hanover Park, IL). Hydroxypropylmethyl cellulose was received from Dow Chemical Company (Midland, MI).

2.2. *In vitro* transport studies

In vitro permeation studies were performed across the human nail plate using Franz diffusion apparatus (Logan Instruments LTD, Somerset, NJ) for 24 h. Sodium fluorescein was used as a model transporting substance. Before performing an experiment, nail plates were soaked in PBS for 2 h. And then they were sandwiched between top and bottom compartments of the Franz diffusion cells with their ventral side facing toward the bottom compartment. The top compartment was filled with 0.5 ml of PBS and the bottom compartment was filled with 5ml of sodium fluorescein solution (1 mg/ml), respectively. Cathodal iontophoresis was performed by fixing cathode and anode electrodes in the bottom and top compartments respectively at a distance of 2 mm from the nail plate. Silver and silver chloride electrodes were used as anode and cathode electrodes and constant direct current (0.5 mA/cm2) was applied across the nail plate using iontophoresis device (Active dose II iontophoresis delivery unit, Transport Pharmaceuticals, Boston, MA). The bottom compartment solution was stirred continuously during the experiment using 3 mm magnetic bars at 600 rpm to distribute the permeated fluorescein dye homogenously. PBS in top compartment was replaced with fresh buffer every two hours. Passive transport studies were performed simultaneously with a similar set up, along

with iontophoresis in which no current was applied. The amount of drug retained in the nail plate was quantified by high-performance liquid chromatography (HPLC).[3,13-15]

2.3. Extraction of sodium fluorescein from the nail plate

After *in vitro* transport studies, the nail plates were detached from adaptor using forceps. The amount of sodium fluorescein retained in the nail plate was extracted by solvent extraction method. The active and lateral diffusion areas of the nail plate were carefully excised using metric punch apparatus. Every nail plate was washed out five times by shaking in ethanol and water alternatively using forceps and then wiped off completely with Kimwipes®. Each nail plate was cut into small pieces and solubilized in 1.5 ml of 1 M sodium hydroxide solution, followed by neutralization with 200 ml of 5 M hydrochloric acid.[42-44] Then, 2 ml of ethyl acetate was added and centrifuged the final mixture at 2000 rpm for 10 min for the complete phase separation of solvent layers. Finally, ethyl acetate layer was collected and evaporated using nitrogen evaporator to get solid crystals of the fluorescein dye.[3,13-15] Then, sodium fluorescein was reconstituted in water and quantified by HPLC.[45]

2.4. Histological studies of the nail plate

After transport studies, nail plates were embedded in the paraffin wax and transverse sections of the nail plates (10 mm) were prepared using Reichert-Jung 820 histocut microtome equipment (GMI, MN). All the images of the transverse sections were taken on 10x magnification using Eclipse 90i Microscope (Nikon, NY).[46]

2.5. *Ex vivo* transport studies in cadaver toe model

Ex vivo transport studies was performed on excised human cadaver toe for three days (8 h/day). Terbinafine hydrochloride was used as a model drug. HPMC gel (2% w/v hydroxypropyl methylcellulose) containing 1% w/v terbinafine hydrochloride was prepared for transport

studies.[24,47] One day before the experiment, cadaver toe was treated with 0.5% w/v gentamycin solution and stored at -20 ºC. Before starting the experiment, toe was thawed at room temperature. HPMC gel was placed at hyponychium region of the toe and covered by an adhesive tape. During the experiment, toe was kept wet using foam sheet. Silver (anode electrode) and silver chloride (cathode electrode) electrodes were placed at the hyponychium and proximal nail fold regions on the toe (Figure 20). Anodal iontophoresis was performed for transport studies. Constant direct current (0.5 mA/cm^2) was applied using iontophoresis device (Active dose II iontophoresis delivery unit, Transport Pharmaceuticals, Boston, MA). Passive permeation studies were performed as a control. The formulation was replaced three-four times during iontophoresis and in passive by breaking the process for 2–3 min. The amount of drug retained in the nail bed, nail plate and nail matrix were extracted and quantified using HPLC method.[3,16]

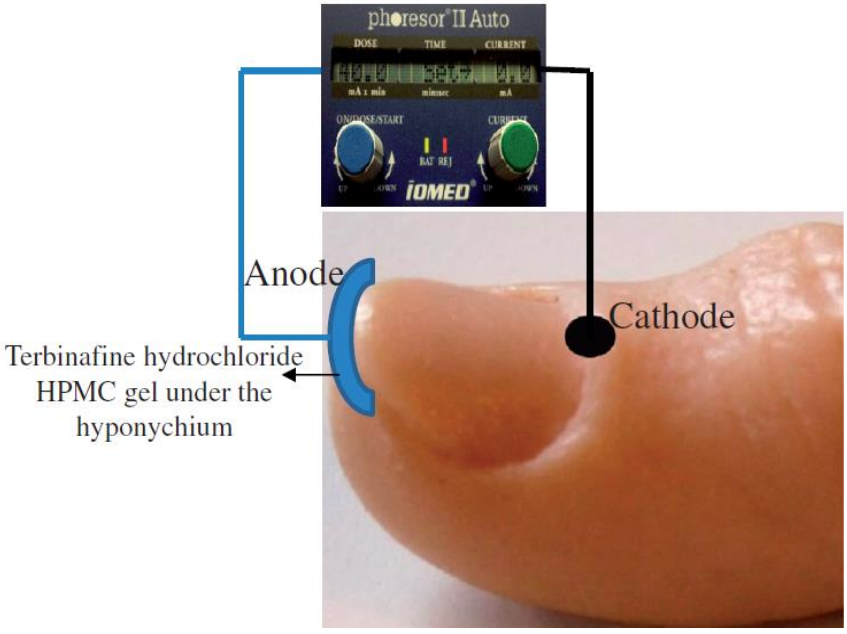


Figure 15. Schematic diagram of *ex vivo* transport studies of terbinafine hydrochloride from hyponychium region

2.6. Extraction of drug from nail plate, nail bed and nail matrix

After transport studies, the nail plate was isolated from the intact toe and washed out five times separately by each of alcohol and water. After every washing, each nail plate was wiped off with Kimwipes® and then solubilized in the 1.5 ml of 1 M sodium hydroxide solution by subjecting under ultra-sonication for 2 h. The alkaline solution was neutralized by adding 200 ml of 5 M hydrochloric acid, followed by adding 3 ml of hexane for the extraction of terbinafine.[42-44] The mixture was centrifuged at 3000 rpm for 15 min, and then, hexane layer was collected. Eventually, 1 ml of 0.5 M sulfuric acid–isopropyl alcohol mixture (85:15) was added to the hexane solution and shaken vigorously for 30 min. After shaking, acidic aqueous layer was carefully separated from the mixture. And then, the amount of drug extracted from the nail plate was quantified using HPLC.[42-44]

After permeation studies, the amount of terbinafine hydrochloride permeated into the nail bed and nail matrix was extracted and quantified following same method discussed earlier.[44]

2.7. Analytical method

High performance liquid chromatography system (HPLC) was used to quantify the amount of terbinafine in the samples. HPLC system consisted of an autosampler (waters 717 plus), Phenomenex C18 (2) 100 R analytical column (4.6 mm × 150 mm, Luna, 5.0 μm), Waters® dual wavelength UV detector (2487) was used. Mobile phase was prepared by combination of two solvents, aqueous phase (0.096 M triethyl amine, 0.183 M orthophosphoric acid) and acetonitrile (60:40). The final pH of the mobile phase was adjusted to pH 2 using orthophosphoric acid. The drug was eluted using isocratic solvent system at a flow rate of 1.0 ml/min at the temperature of 32 ºC. Terbinafine was detected at 224 nm wavelength. To validate the method, calibration curve was prepared from the range of 0.1 to 10 μg /ml (R2 = 0.99).[8]

The amount of sodium fluorescein in the samples was quantified using same HPLC set up (mentioned above) including fluorescence detector. Mobile phase was prepared by mixing acetonitrile, water and triethyl amine (4.0: 5.95: 0.05) at certain ratio. The final pH of the mobile phase was adjusted to pH 3 using orthophosphoric acid. Elution of sodium fluorescein was carried out isocratically at a flow rate of 1 ml/m. The sodium fluorescein was detected at the excitation wavelength of 460 nm and emission wavelength of 515 nm. Retention time of the fluorescein was found at ~9.30 min. Calibration curve was prepared from the range of 0.001 to 10 µg/ml (R2 = 0.99).[45]

2.8. Data Analysis

The statistical analysis of the transport studies was carried out by one-way analysis of variance (ANOVA) test and p values less than 0.05 was considered statistically significant.

3. Results and Discussion

Figure 16. Transverse section of nail plate. [Dorsal layer (DL), Ventral layer (VL)]

The human nail plate is known to consist of three different layers; dorsal, ventral and intermediate. Due to histological similarity between intermediate and ventral layers, only two layers of the nail plate are distinctly identified in the optical microscopic picture shown in Figure

21.[3,11] According to Kabayashi, the dorsal layer of the nail plate acts as the predominant barrier and limits the transportation of drugs into deeper stratums of the nail apparatus.[11] Several other groups have also confirmed that the drug transport into the nail apparatus increases when the dorsal layer of the nail plate is abraded.[37,38,48-51] Therefore, the present work was aimed to investigate the feasibility of utilizing the hyponychium pathway to deliver antifungal drugs directly into the ventral layer of the nail plate and other parts of the nail apparatus. The hyponychium is an area of the nail bed epithelium that underlies free edge of the nail plate.

Iontophoresis technique was used as a tool to improve the delivery and distribution of the drug into the ventral layer of the nail plate. *In vitro* and *ex vivo* transport studies were performed to assess the effectiveness of iontophoresis for enhancing the permeation of drug molecule into the nail plate and other parts of the nail apparatus.

3.1. *In vitro* transport studies

In case of iontophoresis, the active electrode was placed in the bottom compartment and counter electrode was fixed in the top compartment. The amount of sodium fluorescein loaded in the nail plate after application of cathodal iontophoresis was 92.4 ± 7.6 ng/ mg which was ~54 fold more than passive (1.7 ± 0.7 ng/mg) (Figure 22). The amount of sodium fluorescein retained in the peripheral area of the nail plate in the case of iontophoresis (25.5 ± 9.03 ng/mg) was found to be ~30-folds more compared to passive (0.85 ± 0.2 ng/mg) (Figure 23). *In vitro* transport studies have clearly demonstrated that iontophoresis from ventral side of the nail plate might be a potential option for delivery of drugs into the deeper layers of the nail plate.

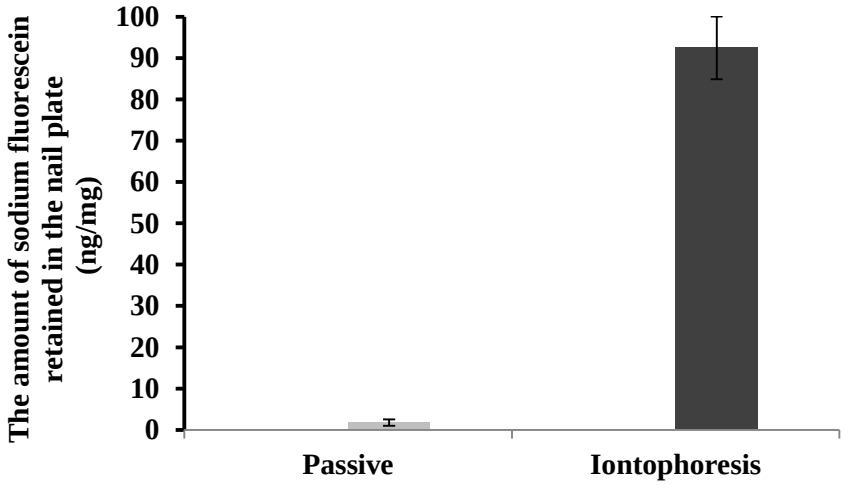


Figure 17. The amount of sodium fluorescein retained in the active diffusion area of the nail plate after 24 h *in vitro* transport studies. The data represent mean ± SD of six determinations.

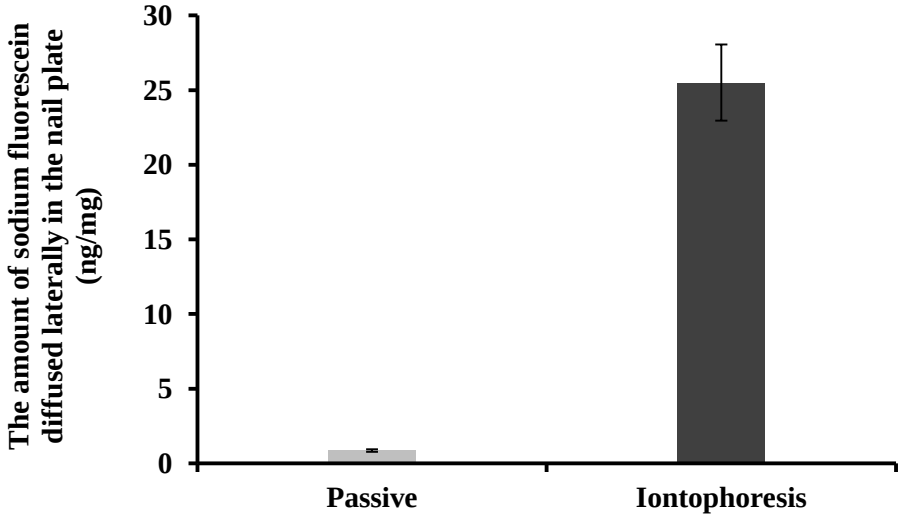


Figure 18. The amount of sodium fluorescein retained in the peripheral area of the nail plate after 24 h *in vitro* transport studies. The data represent mean ± SD of six determinations.

3.2. Histological studies

As the results of histological studies, the penetration depth of sodium fluorescein in the nail plate after application of iontophoresis was found 240 mm of total thickness (254 mm) of the nail plate. In case of passive, the penetration depth was found approximately 69 mm of total thickness of the nail plate (247 mm). Finally, iontophoresis technique improved 4 times more penetration of sodium fluorescein in the nail plate than passive (Figure 24).

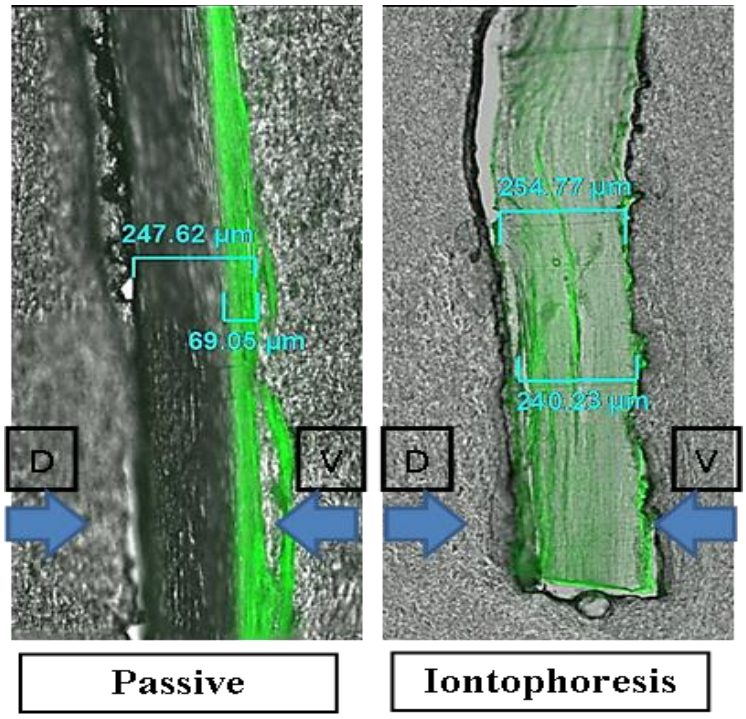


Figure 19. Transverse sections of the nail plates after *in vitro* transport studies of sodium fluorescein

3.3. *Ex vivo* transport studies

Ex vivo studies were performed on the excised cadaver toe model to assess the practical feasibility of iontophoresis from the distal end of the toe. Although the toe model lacks the clearance component, it is anatomically similar to in vivo model and found to be the closest model to clinical investigation. Terbinafine hydrochloride HPMC gel was used to perform *ex vivo* studies. Iontophoresis was carried out for three days (8 h/day). The amount of drug retained

in the nail plate in the case of iontophoresis was 3.43 ± 1.34 mg/mg, which was 20-fold more when compared with passive (0.17 ± 0.10 mg/mg). In case of iontophoresis, the amount of drug found in the nail bed and nail matrix were 1.73 ± 0.124 mg/mg and 0.55 ± 0.22 mg/mg (Figure 25). However, in case of control, there was no detectable amount of terbinafine found in the nail bed and nail matrix.

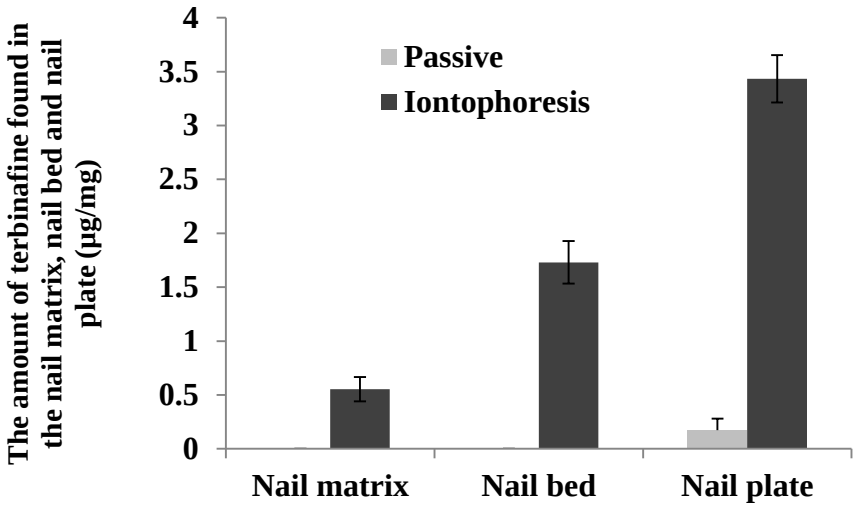


Figure 20. The amount of terbinafine retained in the nail pate, nail bed and nail matrix after *ex-vivo* transport studies for 3 days (8 h/ day). The data represent mean ± SD of three determinations.

4. Conclusions

Iontophoresis from the hyponychium end appears to be a viable option for the delivery of drugs into the nail plate and other parts of nail apparatus. However, this approach is limited by small area of hyponychium available for the application of formulation.

CHAPTER 5: PRE-TREATMENT WITH SOLID MICRONEEDLES FOR UNGUAL AND TRANS-UNGUAL DELIVERY OF DRUGS

1.0 Introduction

Microneedle pre-treatment is a novel approach which has already been used to deliver small and large molecules into the skin in a minimally invasive manner.[17-19,52] The application of microneedles has not been explored before to improve nail delivery of drugs.[18,53] In this project, solid microneedle pre-treatment was investigated to enhance the delivery of drugs into the nail apparatus. *In vitro* permeation studies were performed across the human nail plate pretreated with 0.5 mm solid titanium microneedles. Sodium fluorescein was used as a model dye. After permeation studies, the amount of sodium fluorescein found into and permeated across nail plate was quantified using HPLC method. Microscopic studies were performed to show the distribution of sodium fluorescein in nail plate.

2.0 Materials and Methods:

2.1 Materials

Dermal stamp (0.5 mm) was purchased from GHgate. Sodium fluorescein was purchased from Sigma-Aldrich (St. Louis, MO). Human nails were procured from Science Care (Phoenix, AZ). The solvents were bought from Fisher Chemicals (Hanover Park, IL).

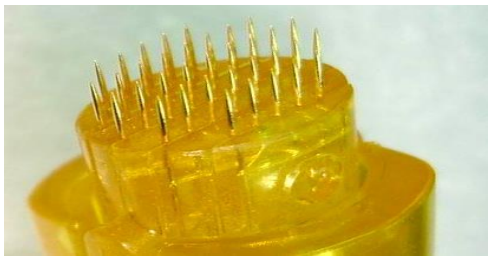


Figure 21. Image of titanium microneedles of dermal stamp

2.*2 In vitro* permeation studies:

In vitro transport studies were performed across the human nail plate using Franz diffusion apparatus (Logan Instruments Ltd, Somerset NJ) for 7 days. Sodium fluorescein was used as a model dye for transport studies. Before performing an experiment, nail plates were hydrated in phosphate buffer saline (PBS) for 2 h. Nail plate was pretreated with 0.5 mm long titanium microneedles and then sandwiched between donor and receiver compartments using nail adapter (PermeGear, Hellertown, PA) having 0.3 cm^2 active diffusion area. Nail plate with no microneedles pretreatment was used as a control. Donor compartment was filled with 0.5 ml of 1 mg/ml sodium fluorescein solution in PBS. Receiver compartment was filled with 5ml of phosphate buffer saline. The solution in receiver compartment was stirred continuously during the experiment using 3 mm magnetic bars at 600 rpm to distribute the permeated fluorescein dye homogenously. Samples were collected at definite period of time from receiver compartment. The amount of sodium fluorescein contained in samples was quantified using HPLC method.[3,13-15]

2.3 Extraction of sodium fluorescein from the nail plate

After *in vitro* transport studies, nail plates were separated from an adaptor using forceps. Active diffusion area of nail plate was excised using 0.3 cm^2 metric dermal punch. Then, nail plates were washed 5 times with 50% of ethanol and wiped off with Kimwipes® to remove the extra

amount of sodium fluorescein presented on its outer surface. The amount of sodium fluorescein retained in nail plate was extracted using solvent extraction method. Each nail plate was weighed accurately before solubilizing in 1.5 ml of 1 M sodium hydroxide solution. Hydrochloric acid (5 M, 200 µl) was added to neutralize alkaline solution. Finally, ethyl acetate (2 ml) was added as an extraction solvent and the mixture was centrifuged at 2000 rpm for 10 min. After complete phase separation of solvent layers, ethyl acetate layer was collected and evaporated using nitrogen evaporator to get solid crystals of fluorescein dye.[3, 13] The amount of sodium fluorescein was quantified by HPLC.[45]

The amount of drug diffused in peripheral area of nail plate was extracted and quantified following same method which was mention above in same section 2.3.[45]

2.4. Microscopic studies of solid microneedles pretreated nail plates:

Microscopic studies were performed to show the distribution of sodium fluorescein in nail plate. In this case, *in vitro* permeation studies were performed for 24 h and then active diffusion area of nail plate was excised using dermal metric punch. All nail plates were washed with 90% alcohol and wiped off with Kimwipes® to remove the extra amount of sodium fluorescein presented on its surface. Finally, nail plate was embedded in the paraffin wax. Reichert-Jung 820 histocut microtome equipment (GMI, MN, USA) was used to prepare transverse and horizontal sections (10 µm) of nail plates. All the images of transverse and horizontal sections were taken using Eclipse 90i Microscope (Nikon, NY, USA) at 10X magnification.[45]

2.5. Analytical method:

High performance liquid chromatography system (HPLC) was used to quantify the amount of sodium fluorescein presented in the samples. HPLC system consists of an autosampler (waters 717 plus), Phenomenex C18 (2) 100 R analytical column (4.6 mm × 150 mm, Luna, 5.0 µm),

fluorescence detector. Mobile phase was prepared by mixing acetonitrile, water and triethyl amine (4.0: 5.95: 0.05) at certain ratio. The final pH of the mobile phase was adjusted to pH 3 using orthophosphoric acid. Elution of sodium fluorescein was carried out isocratically at a flow rate of 1 ml/min. The sodium fluorescein was detected at the excitation wavelength of 460 nm and emission wavelength of 515 nm. Calibration curve was prepared from the range of 0.001 to 10 µg/ml ($R^2 = 0.99$).[45]

3.0 Results and discussion

Ungual and trans-ungual delivery of drugs is restricted due to tough nature of dorsal layer of the nail plate.[3,8-10] In previous literatures, several passive and active techniques have been reported to disruption the dorsal layer of nail plate to improve the transportation of drugs.[12] Although, solid microneedles are widely used for the delivery of small and larger molecules into the skin, its application for nail delivery of drugs have not been investigated yet. Therefore, in this project, the application of microneedles was investigated to improve the permeation of drugs into and across nail plate. Different length of solid microneedles (0.2 mm, 0.3 mm, 0.5 mm) were tried to create holes deeper enough to ventral layer of nail plate. After all the observations, 0.5 mm microneedles were found to be more efficient to perform further studies.

***In vitro* permeation studies:**

As a result of *in vitro* permeation studies, the amount of sodium fluorescein found in receiver compartment was 86.2±13.4 ng/cm² which was ~123 fold more compared to control (0.7±0.28 ng/cm²). The amount of sodium fluorescein retained in nail plate in case of microneedles (0.4±0.01 µg/mg) was found to be ~4 fold more related to control (0.10±0.01 µg/mg). The amount of sodium fluorescein diffused in the peripheral area of nail plate was 1.17±0.07 µg/mg which was found to be ~3 fold more compared to control (0.371.6±0.04 µg/mg).

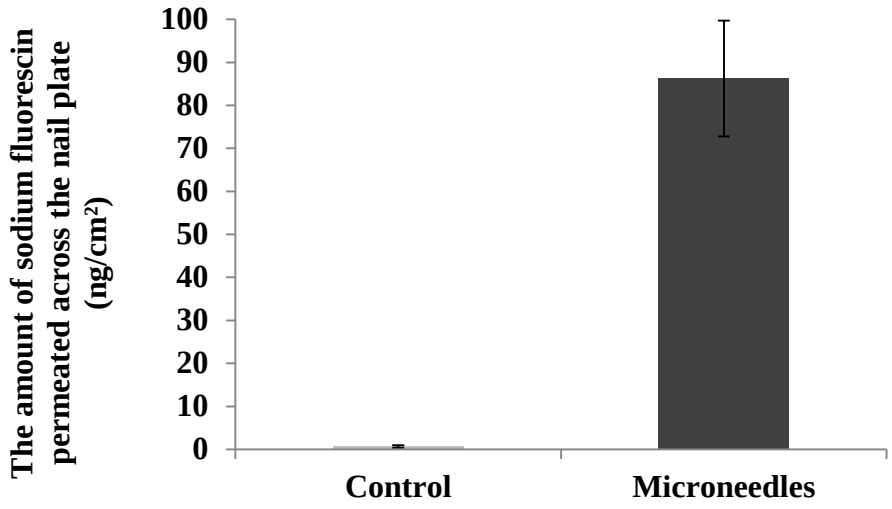


Figure 22. The amount of sodium fluorescein permeated across the nail plate after 7 days of in *vitro* transport studies (μg/cm^2). The data represent mean ± SD of six determinations.

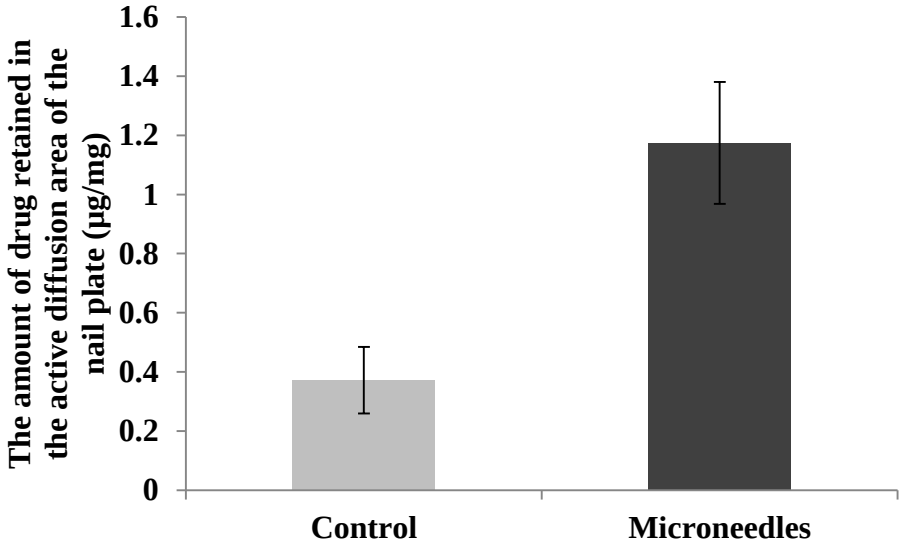


Figure 23. The amount of sodium fluorescein retained in the nail plate after 7 days of *in vitro* transport studies. The data represent mean ± SD of six determinations.

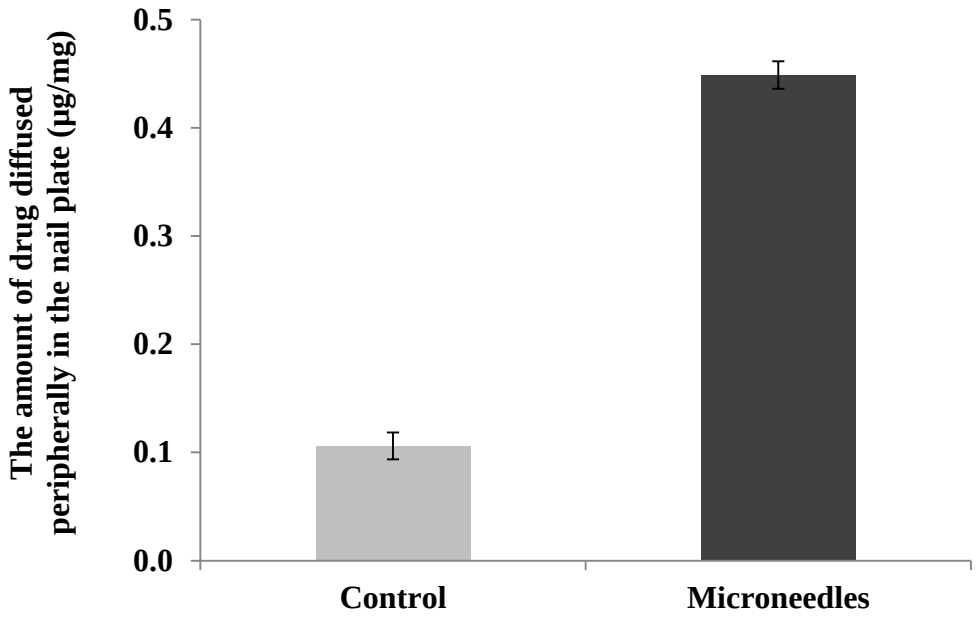


Figure 24. The amount of sodium fluorescein found in peripheral area of nail plate after 7 days of *in vitro* transport studies. The data represent mean ± SD of six determinations.

3.2 Microscopic studies:

Titanium microneedles created approximately 62 pixels (~16 µm) average diameter of holes in nail plate. After 24 h of *in vitro* studies, nail plates were cut in approximately 10 µm thickness of transverse and horizontal sections. The distribution of sodium fluorescein was imaged at 10x magnification using Eclipse 90i Microscope. In case of microneedles pre-treatment, images showed the distribution of sodium fluorescein in all three layers of the nail plate. However, fluorescein dye was observed only on top layer of nail plate in case of control.

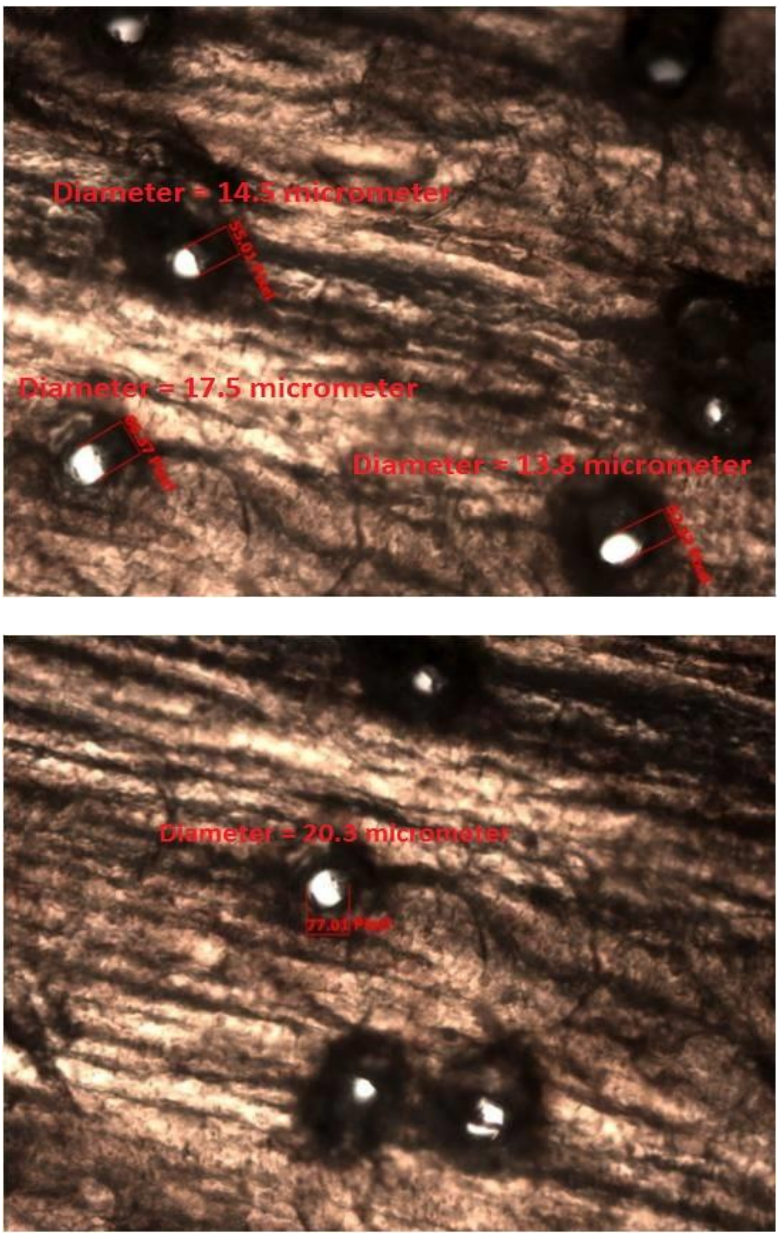
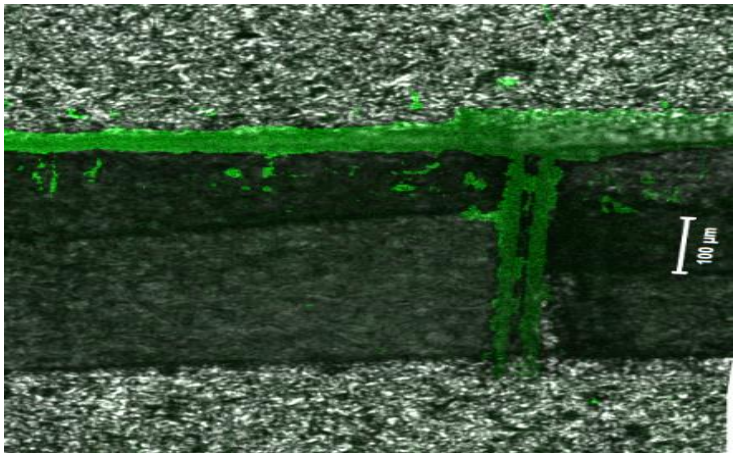
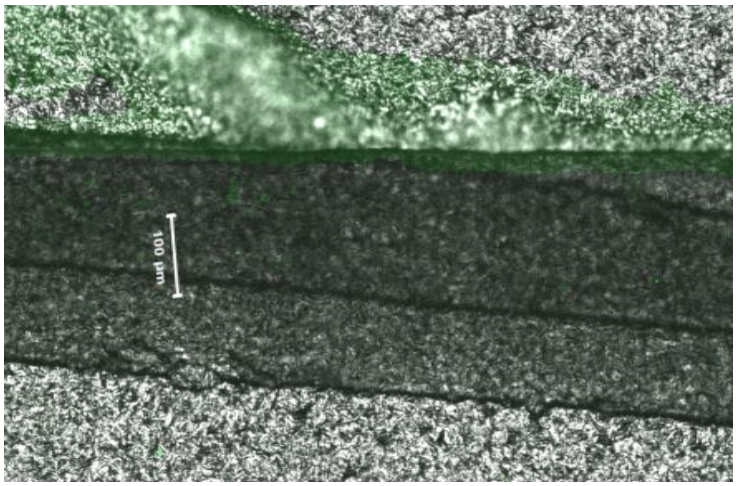


Figure 25. Horizontal sections of nail plate after treatment with solid titanium microneedles. The image was taken from dorsal layer of nail plate.



Transverse sections of microneedles pre-treated nail plate



Transverse sections of nail plate (Control)

Figure 26. Transverse sections of nail plates after 24 h of *in vitro* transport studies of sodium fluorescein.

4.0 Conclusion: Pre-treatment of nail plate with solid microneedles was able to deliver significant amount of sodium fluorescein into and across the nail plate. It is evident that pre-treatment with microneedles could be a potential option for ungual and trans-ungual delivery of drugs for the treatment of nail diseases.

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VITA

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Profile

- Accomplished PhD in Pharmaceutics with research expertise in novel pharmaceutical pre-formulation and formulation development, design, testing and analysis with special focus on transdermal, topical and transungual (Nail) drug delivery systems.
- Proficiency in performing cell line (Dermal fibroblast and keratinocytes) and dermal toxicity studies.
- Special skills in protein formulation development and its isolation, purification and characterization studies.
- Implemented pharmacokinetics and pharmacodynamic studies in rat models and rabbit.
- Innovative researcher with exceptional skills in project management, technology evaluation, testing, and analysis.

Education

2015-Now	Principal Formulation Scientist in Tioga Research, Inc
2011-16	Ph. D. Candidate
(GPA-3.71/4.0)	Department of Pharmaceutics and Drug Delivery,
	University of Mississippi
	Graduate Advisor: Dr. S. Narasimha Murthy
08/05 – 06/09	Bachelor of Pharmacy
(GPA-3.8/4.0)	Dr. Hari Singh Gour Central University, Sagar, India

Work Experiences

Graduate Research Assistant, Pharmaceutics

University of Mississippi, University, MS (2011 – Present)

- Experienced in working on industrial project, collaborated with ARNO therapeutics for the transungual (Nail) delivery of a novel molecule AR-12. Clinical studies were performed on finger and toe nails of human volunteers. (http://www.arnothera.com/pr150919.html)
- Performed cell culture (fibroblast and keratinocyte) and dermal toxicity studies in my projects. I have also worked on keratinase enzyme formulation development and collagen protein isolation, purification and characterization studies.

- Developed a novel epidermis preparation method which is a critical step in performing transdermal and topical permeation studies.
- Carried out the trans-nail and transdermal delivery of novel drug molecules using passive (chemical enhancers) and physical techniques (Iontophoresis, Electrophoresis and Microneedles).
- Conducted pharmacokinetic studies in plasma, skin and brain utilizing intravenous and transdermal application methods in rat models.
- Overseen all laboratory activities and supervised lab personnel.

Laboratory & Technical Experience

- Development of pre-formulation and formulation of topical, transdermal and trans-ungual dosage forms utilizing:
    - *In vitro* drug permeation studies across skin, nail and nasal tissues using vertical & horizontal diffusion apparatus.
    - Passive (chemical penetration enhancers) and physical drug delivery techniques (Iontophoresis, Ultrasonic, Microneedles and Electrophoresis)
    - *In vivo* drug permeation studies using rat and rabbit as the animal models. Clinical studies were carried out on healthy human volunteers.
- Quantitative and characterization assay method development and validation using:
    - Analytical techniques such as HPLC, UV-Visible and Fluorescent spectroscopic techniques and characterization techniques such as FTIR, Differential scanning calorimetry (DSC) and X ray diffraction (XRD) electron scanning microscopy (SEM)
- Protein isolation, purification, characterization using:
    - Ion-Exchange gel chromatography, Size Exclusion gel chromatography, SDS page, western blotting, Circular dichroism, ELISA assay
- Cell line (fibroblast and keratinocyte cells) and dermal toxicity studies by performing
    - MTT assay, estimation of cytokines levels, irritation studies

Media Release

Arno therapeutics, iAdweek, Virmmac

Editorial board member

Journal of Biotechnology & Biomaterials

Judge and reviewer for journals

AAPS Pharma tek, Aging, Journal of bioequivalence and bioavailability, Journal of drug delivery science and technology, biological sciences, BMC research notes, Current drug delivery, Drug discovery and industrial pharmacy, Expert opinion on drug delivery, International journal of pharmaceutical compounding, Plose one, Journal of Biotechnology & Biomaterials, Journal of Biomedical Engineering and Medical Devices

Invited speaker

Biocom: Skin-Applied Products: From Concept to Approvals in San Diego

Research Publications

- **Kushwaha A,** Jacob M, Shivakumar HN, Hiremath S, Aradhya S, Repka MA, Murthy SN. Trans-ungual delivery of itraconazole hydrochloride by iontophoresis. Drug Dev Ind Pharm 2014; 8:1-5.
- **Kushwaha A,** Murthy RN, Murthy SN, Elkeeb R. Hui X, Maibach HI. Emerging therapies for the treatment of ungual onychomycosis. Drug Dev Ind Pharm 2015; 22:1-7.
- **Kushwaha A,** Murthy SN. Iontophoresis for drug delivery into the nail apparatus: Exploring hyponychium as a site of delivery. Drug Dev Ind Pharm 2016; 23:1-5.
- Manda P**, Kushwaha A,** Murthy SN. Delivery of ziconotide to cerebrospinal fluid via intranasal pathway for the treatment of chronic pain. J Control Release 2016; 224:69-76.
- **Kushwaha A,** Murthy SN. Trans-ungual delivery of apremilast for the treatment of nail psoriasis. (*Manuscript under communication with AAPS Journal*)
- **Kushwaha A,** Murthy SN. Trans-ungual delivery of AR-12, a novel antifungal drug. (*Manuscript under communication with Journal of Pharmaceutical Sciences*)
- **Kushwaha A,** Murthy SN. Preparation of epidermis using a novel salt stripping method. (*Manuscript under preparation*)
- **Kushwaha A,** Murthy SN. Pre-treatment of solid microneedles for trans-ungual delivery of drugs. (*Manuscript under communication with Drug Dev Ind Pharm*).
- **Kushwaha A,** Murthy SN. A novel topical treatment of hypertrophic scar. (Manuscript under preparation).
- **Kushwaha A,** Murthy SN. TransScreening of permeation enhancers for terbinafine hydrochloride. (Manuscript under preparation).

Selected Presentations at National and International Conferences

- **Kushwaha A,** Shivakumar H.N, Murthy S.N. Trans-ungual iontophoretic delivery of itraconazole. AAPS Annual meeting and Exposition, Chicago, IL, October 2012.
- **Kushwaha A,** Murthy S.N. Topical Treatment of Hypertrophic Scars. AAPS Annual meeting and Exposition, Chicago, IL, October 2012.
- **Kushwaha A,** Shivakumar H.N, Murthy S.N. Bioadhesive film forming formulation for topical delivery of drugs. AAPS Annual meeting and Exposition, San Antonio, TX, November 2013.
- **Kushwaha A,** Shivakumar H.N, Murthy S.N. Conductive topical nail lacquer for transungual iontophoresis. AAPS Annual meeting and Exposition, San Antonio, TX, November 2013.
- **Kushwaha A,** Jacob M.R, Murthy S.N. Continuous versus discontinuous application modes of iontophoresis on the trans-ungual delivery of itraconazole. AAPS Annual meeting and Exposition, San Antonio, TX, November 2013.
- **Kushwaha A,** Shivakumar H.N, Murthy S.N. Losartan loaded hydrogel microparticles for delivery of drugs to Chronic Wounds. AAPS Annual meeting and Exposition, San Antonio, TX, November 2013.
- **Kushwaha A,** Murthy S.N. Poloxamer facilitates the recovery of functional properties of stratum corneum impaired due to thermal exposure. AAPS Annual meeting and Exposition, San Antonio, TX, November 2013.

- **Kushwaha A,** Shivakumar H.N, Murthy S.N. Reverse iontophoresis for Trans-Ungual Drug Delivery. AAPS Annual meeting and Exposition, San Antonio, TX, November 2013.
- **Kushwaha A,** Manda P, Murthy S.N. Intranasal delivery of fluorocytosine. AAPS Annual meeting and Exposition, San diego, CA, November 2014.
- **Kushwaha A,** Manda P, Murthy S.N. A topical controlled release formulation for the treatment of postherpatic neuralgia. AAPS Annual meeting and Exposition, San diego, CA, November 2014.
- **Kushwaha A,** Sharma P, Murthy S.N Trans-ungual Delivery of Apremilast for the Treatment of Nail Psoriasis. AAPS Annual meeting and Exposition, Orlando, FL, October 2015.
- **Kushwaha A,** Murthy S.N Preparation of Epidermis Using a Novel Salt Stripping Method. AAPS Annual meeting and Exposition, Orlando, FL, October 2015.
- **Kushwaha A,** Sharma P, Murthy S.N Trans-ungual Delivery of AR-12, a novel antifungal drug. AAPS Annual meeting and Exposition, Orlando, FL, October 2015.

Software Programs

Ubuntu operating system, Mathematica, Graph Pad prism, MS Office (Excel, Word and Power point), SPSS statistical software.

Honors / Awards

- Graduate Student Council Research Award, the University of Mississippi, 2013.
- COBRE Grant Award (NIH): Center of Research Excellence in Natural Products Neuroscience Fellowship, 2014.
- COBRE Grant Award (NIH): Center of Research Excellence in Natural Products Neuroscience Fellowship, 2015.
- RhoChi, Honors society award, 2013.

Professional Membership

- American Association of Pharmaceutical Scientists (AAPS).
- Dermatopharmaceutics focus group
- Physical pharmacy and biopharmaceutics section (SRDG).
- Formulation Design and Development Section, AAPS.